

(11) **EP 0 693 687 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
24.01.1996 Bulletin 1996/04

(51) Int. Cl.⁶: **G01N 33/576, C07K 14/18**

(21) Application number: 95114016.9

(22) Date of filing: 03.04.1991

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(30) Priority: 04.04.1990 US 504352

(62) Application number of the earlier application in
accordance with Art. 76 EPC: 91302910.4

(71) Applicant: **CHIRON CORPORATION**
Emeryville, California 94608 (US)

(72) Inventors:
• **Houghton, Michael**
Danville, California 94526 (US)

• **Choo, Qui-Lim**
El Cerrito, California 94530 (US)
• **Kuo, George**
San Francisco, California 94112 (US)

(74) Representative: **Brasnett, Adrian Hugh**
London WC1R 5LX (GB)

Remarks:

This application was filed on 07 - 09 - 1995 as a
divisional application to the application mentioned
under INID code 60.

z(54) Combinations of hepatitis C virus (HCV) antigens for use in immunoassays for anti-HCV antibodies

(57) Combinations of HCV antigens that have a broader range of immunological reactivity than any single HCV antigen. The combinations consist of an antigen from C domain of the HCV polyprotein, and one additional HCV antigen from the S domain, optionally with an additional antigen from either the NS3 domain, the NS4 domain, or the NS5 domain, and are in the form of a fusion protein, a simple physical mixture, or the individual antigens commonly bound to a solid matrix.

EP 0 693 687 A1

DescriptionTechnical Field

5 The present invention is in the field of immunoassays for HCV (previously called Non-A, Non-B hepatitis virus). More particularly, it concerns combinations of HCV antigens that permit broad range immunoassays for anti-HCV antibodies.

Background

10 The disease known previously as Non-A, Non-B hepatitis (NANBH) was considered to be a transmissible disease or family of diseases that were believed to be viral-induced, and that were distinguishable from other forms of viral-associated liver diseases, including that caused by the known hepatitis viruses, i.e., hepatitis A virus (HAV), hepatitis B virus (HBV), and delta hepatitis virus (HDV), as well as the hepatitis induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV). NANBH was first identified in transfused individuals. Transmission from man to chimpanzee and serial
 15 passage in chimpanzees provided evidence that NANBH was due to a transmissible infectious agent or agents. Epidemiologic evidence suggested that there may be three types of NANBH: a water-borne epidemic type; a blood-borne or parenterally transmitted type; and a sporadically occurring (community acquired) type. However, until recently, no transmissible agent responsible for NANBH had been identified, and clinical diagnosis and identification of NANBH had been accomplished primarily by exclusion of other viral markers. Among the methods used to detect putative NANBH antigens and antibodies were agar-gel diffusion, counterimmunoelectrophoresis, immunofluorescence microscopy, immune elec-
 20 tron microscopy, radioimmunoassay, and enzyme-linked immunosorbent assay. However, none of these assays proved to be sufficiently sensitive, specific, and reproducible to be used as a diagnostic test for NANBH.

In 1987, scientists at Chiron Corporation (the owner of the present application) identified the first nucleic acid definitively linked to blood-borne NANBH. See, e.g., EPO Pub. No. 318,216; Houghton et al., *Science* 244:359 (1989). These
 25 publications describe the cloning of an isolate from a new viral class, hepatitis C virus (HCV), the prototype isolate described therein being named "HCV1." HCV is a Flavi-like virus, with an RNA genome.

U.S. Patent Application Serial No. 456,637 (Houghton et al.), incorporated herein by reference, describes the preparation of various recombinant HCV polypeptides by expressing HCV cDNA and the screening of those polypeptides for immunological reactivity with sera from HCV patients. That limited screening showed that at least five of the polypep-
 30 tides tested were very immunogenic; specifically, those identified as 5-1-1, C100, C33c, CA279a, and CA290a. Of these five polypeptides, 5-1-1 is located in the putative NS4 domain; C100 spans the putative NS3 and NS4 domains; C33c is located within the putative NS3 domain and CA279a and CA290a are located within the putative C domain. The screening also showed that no single polypeptide tested was immunologically reactive with all sera. Thus, improved tests, which react with all or more samples from HCV positive individuals, are desirable.

Disclosure of the Invention

Applicants have carried out additional serological studies on HCV antigens that confirm that no single HCV polypep-
 40 tide identified to date is immunologically reactive with all sera. This lack of a single polypeptide that is universally reactive with all sera from individuals with HCV may be due, *inter alia*, to strain-to-strain variation in HCV epitopes, variability in the humoral response from individual-to-individual and/or variation in serology with the state of the disease.

These additional studies have also enabled applicants to identify combinations of HCV antigens that provide more efficient detection of HCV antibodies than any single HCV polypeptide.

Accordingly, one aspect of this invention is a combination of HCV antigens comprising:

- 45 (a) a first HCV antigen from the C domain; and
 (b) at least one additional HCV antigen selected from the group consisting of

- 50 (i) an HCV antigen from the NS3 domain;
 (ii) an HCV antigen from the NS4 domain;
 (iii) an HCV antigen from the S domain; and
 (iv) an HCV antigen from the NS5 domain.

In one embodiment, the combination of HCV antigens is in the form of a fusion protein comprised of the antigens.
 55 In an alternative embodiment, the combination of antigens is in the form of the individual antigens bound to a common solid matrix. In still another embodiment, the combination of antigens is in the form of a mixture of the individual antigens.

Another aspect of the invention is a method for detecting antibodies to HCV in a mammalian body component suspected of containing said antibodies comprising contacting said body component with the above-described combi-

nation of HCV antigens under conditions that permit antibody-antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

Another aspect of the invention is a method for detecting antibodies to HCV in a mammalian body component suspected of containing said antibodies comprising contacting said body component with a panel of HCV antigens, simultaneously or sequentially, comprising

- (a) a first HCV antigen from the C domain; and
- (b) at least one additional HCV antigen selected from the group consisting of

- (i) an HCV antigen from the NS3 domain;
- (ii) an HCV antigen from the NS4 domain;
- (iii) an HCV antigen from the S domain; and
- (iv) an HCV antigen from the NS5 domain

under conditions that permit antibody-antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

Another aspect of the invention is a kit for carrying out an assay for detecting antibodies to HCV in a mammalian body component suspected of containing said antibodies comprising in packaged combination

- (a) said combination of HCV antigens;
- (b) standard control reagents; and
- (c) instructions for carrying out the assay.

Brief Description of the Drawings

In the drawings:

Figure 1 is the nucleotide sequence of the cDNA sense and anti-sense strand for the HCV polyprotein and the amino acid sequence encoded by the sense strand.

Figure 2 is a schematic of the amino acid sequence of Figure 1 showing the putative domains of the HCV polypeptide.

Modes for Carrying Out the Invention

Definitions

"HCV antigen" intends a polypeptide of at least about 5 amino acids, more usually at least about 8 to 10 amino acids that defines an epitope found in an isolate of HCV. Preferably, the epitope is unique to HCV. When an antigen is designated by an alphanumeric code, the epitope is from the HCV domain specified by the alphanumeric.

"Synthetic" as used to characterize an HCV antigen intends that the HCV antigen has either been isolated from native sources or man-made such as by chemical or recombinant synthesis.

"Domains" intends those segments of the HCV polyprotein shown in Figure 2 which generally correspond to the putative structural and nonstructural proteins of HCV. Domain designations generally follow the convention used to name Flaviviral proteins. The locations of the domains shown in Figure 2 are only approximate. The designations "NS" denotes "nonstructural" domains, while "S" denotes the envelope domain, and "C" denotes the nucleocapsid or core domain.

"Fusion polypeptide" intends a polypeptide in which the HCV antigen(s) are part of a single continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or be separated by intervening amino acid sequences. The fusion polypeptides may also contain amino acid sequences exogenous to HCV.

"Common solid matrix" intends a solid body to which the individual HCV antigens or the fusion polypeptide comprised of HCV antigens are bound covalently or by noncovalent means such as hydrophobic adsorption.

"Mammalian body component" intends a fluid or tissue of a mammalian individual (e.g., a human) that commonly contains antibodies produced by the individual. Such components are known in the art and include, without limitation, blood, plasma, serum, spinal fluid, lymph fluid, secretions of the respiratory, intestinal or genitourinary tracts, tears, saliva, milk, white blood cells, and myelomas.

"Immunologically reactive" means that the antigen in question will react specifically with anti-HCV antibody commonly present in a significant proportion of sera from individuals infected with HCV.

"Immune complex" intends the combination or aggregate formed when an antibody binds to an epitope on an antigen.

Combinations of HCV Antigens

Figure 2 shows the putative domains of the HCV polyprotein. The domains from which the antigens used in the combinations derive are: C, S (or E), NS3, NS4, and NS5. The C domain is believed to define the nucleocapsid protein of HCV. It extends from the N-terminal of the polyprotein to approximately amino acid 120 of Figure 1. The S domain is believed to define the virion envelope protein, and possibly the matrix (M) protein, and is believed to extend from approximately amino acid 120 to amino acid 400 of Figure 1. The NS3 domain extends from approximately amino acid 1050 to amino acid 1640 and is believed to constitute the viral protease. The NS4 domain extends from the terminus of NS3 to approximately amino acid 2000. The function of the NS4 protein is not known at this time. Finally, the NS5 domain extends from about amino acid 2000 to the end of the polyprotein and is believed to define the viral polymerase.

The sequence shown in Figure 1 is the sequence of the HCV1 isolate. It is expected that the sequences of other strains of the blood-borne HCV may differ from the sequence of Figure 1, particularly in the envelope (S) and nucleocapsid (C) domains. The use of HCV antigens having such differing sequences is intended to be within the scope of the present invention, provided, however, that the variation does not significantly degrade the immunological reactivity of the antigen to sera from persons infected with HCV.

In general, the HCV antigens will comprise entire or truncated domains, the domain fragments being readily screened for antigenicity by those skilled in the art. The individual HCV antigens used in the combination will preferably comprise the immunodominant portion (i.e., the portion primarily responsible for the immunological reactivity of the polypeptide) of the stated domain. In the case of the C domain it is preferred that the C domain antigen comprise a majority of the entire sequence of the domain. The antigen designated C22 (see Example 4, *infra*), is particularly preferred. The S domain antigen preferably includes the hydrophobic subdomain at the N-terminal end of the domain. This hydrophobic subdomain extends from approximately amino acid 199 to amino acid 328 of Figure 1. The HCV antigen designated S2 (see Example 3, *infra*), is particularly preferred. Sequence downstream of the hydrophobic subdomain may be included in the S domain antigen if desired.

A preferred NS3 domain antigen is the antigen designated C33c. That antigen includes amino acids 1192 to 1457 of Figure 1. A preferred NS4 antigen is C100 which comprises amino acids 1569 to 1931 of Figure 1. A preferred NS5 antigen comprises amino acids 2054 to 2464 of Figure 1.

The HCV antigen may be in the form of a polypeptide composed entirely of HCV amino acid sequence or it may contain sequence exogenous to HCV (i.e., it may be in the form of a fusion protein that includes exogenous sequence). In the case of recombinantly produced HCV antigen, producing the antigen as a fusion protein such as with SOD, alpha-factor or ubiquitin (see commonly owned U.S. Pat. No. 4,751,180, U.S. Pat. No. 4,870,008 and U.S. Pat. Application Serial. No. 390,599, filed 7 August 1989, the disclosures of which are incorporated herein, which describe expression of SOD, alpha-factor and ubiquitin fusion proteins) may increase the level of expression and/or increase the water solubility of the antigen. Fusion proteins such as the alpha-factor and ubiquitin fusion are processed by the expression host to remove the heterologous sequence. Alpha-factor is a secretion system, however, while ubiquitin fusions remain in the cytoplasm.

Further, the combination of antigens may be produced as a fusion protein. For instance, a continuous fragment of DNA encoding C22 and C33c may be constructed, cloned into an expression vector and used to express a fusion protein of C22 and C33c. In a similar manner fusion proteins of C22 and C100; C22 and S2; C22 and an NS5 antigen; C22, C33c, and S2; C22, C100 and S2, and C22, C33c, C100, and S2 may be made. Alternative fragments from the exemplified domain may also be used.

Preparation of HCV Antigens

The HCV antigens of the invention are preferably produced recombinantly or by known solid phase chemical synthesis. They may, however, also be isolated from dissociated HCV or HCV particles using affinity chromatography techniques employing antibodies to the antigens.

When produced by recombinant techniques, standard procedures for constructing DNA encoding the antigen, cloning that DNA into expression vectors, transforming host cells such as bacteria, yeast, insect, or mammalian cells, and expressing such DNA to produce the antigen may be employed. As indicated previously, it may be desirable to express the antigen as a fusion protein to enhance expression, facilitate purification, or enhance solubility. Examples of specific procedures for producing representative HCV antigens are described in the Examples, *infra*, and in parent application Serial No. 456,637.

Formulation of Antigens for Use in Immunoassay

The HCV antigens may be combined by producing them in the form of a fusion protein composed of two or more of the antigens, by immobilizing them individually on a common solid matrix, or by physically mixing them. Fusion proteins of the antigen may also be immobilized on (bound to) a solid matrix. Methods and means for covalently or noncovalently

binding proteins to solid matrices are known in the art. The nature of the solid surface will vary depending upon the assay format. For assays carried out in microtiter wells, the solid surface will be the wall of the well or cup. For assays using beads, the solid surface will be the surface of the bead. In assays using a dipstick (i.e., a solid body made from a porous or fibrous material such as fabric or paper) the surface will be the surface of the material from which the dipstick is made. In agglutination assays the solid surface may be the surface of latex or gelatin particles. When individual antigens are bound to the matrix they may be distributed homogeneously on the surface or distributed thereon in a pattern, such as bands so that a pattern of antigen binding may be discerned.

Simple mixtures of the antigens comprise the antigens in any suitable solvent or dispersing medium.

10 Assay Formats Using Combinations of Antigens

The HCV antigens may be employed in virtually any assay format that employs a known antigen to detect antibodies. A common feature of all of these assays is that the antigen is contacted with the body component suspected of containing HCV antibodies under conditions that permit the antigen to bind to any such antibody present in the component. Such conditions will typically be physiologic temperature, pH and ionic strength using an excess of antigen. The incubation of the antigen with the specimen is followed by detection of immune complexes comprised of the antigen.

Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. Protocols may, for example, use solid supports, or immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the immune complex are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

The immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format, and of a standard or competitive type. In a heterogeneous format, the polypeptide is typically bound to a solid matrix or support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates), polyvinylidene fluoride (known as Immulon™), diazotized paper, nylon membranes, activated beads, and Protein A beads. For example, Dynatech Immulon™ 1 or Immulon™ 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptides is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are known in the art.

In a homogeneous format, the test sample is incubated with the combination of antigens in solution. For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

In a standard format, the amount of HCV antibodies forming the antibody-antigen complex is directly monitored. This may be accomplished by determining whether labeled anti-xenogenic (e.g., anti-human) antibodies which recognize an epitope on anti-HCV antibodies will bind due to complex formation. In a competitive format, the amount of HCV antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing ligand) in the complex.

Complexes formed comprising anti-HCV antibody (or, in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For example, unlabeled HCV antibodies in the complex may be detected using a conjugate of anti-xenogenic Ig complexed with a label, (e.g., an enzyme label).

In an immunoprecipitation or agglutination assay format the reaction between the HCV antigens and the antibody forms a network that precipitates from the solution or suspension and forms a visible layer or film of precipitate. If no anti-HCV antibody is present in the test specimen, no visible precipitate is formed.

The HCV antigens will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain in separate containers the combination of antigens (either already bound to a solid matrix or separate with reagents for binding them to the matrix), control antibody formulations (positive and/or negative), labeled antibody when the assay format requires same and signal generating reagents (e.g., enzyme substrate) if the label does not generate a signal directly. Instructions (e.g., written, tape, VCR, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

The following examples are intended to illustrate the invention and are not intended to limit the invention in any manner.

55 Example 1: Synthesis of HCV Antigen C33c

HCV antigen C33c contains a sequence from the NS3 domain. Specifically, it includes amino acids 1192-1457 of Figure 1. This antigen was produced in bacteria as a fusion protein with human superoxide dismutase (SOD) as follows. The vector pSODc1 (Steiner et al. (1986), J. Virol. 58:9) was digested to completion with EcoRI and BamHI and the

resulting EcoRI, BamHI fragment was ligated to the following linker to form pcf1EF:

GATC CTG GAA TTC TGA TAA

GAC CTT AAG ACT ATT TTA A

A cDNA clone encoding amino acids 1192-1457 and having EcoRI ends was inserted into pcf1EF to form pcf1EF/C33c.

This expression construct was transformed into D1210 *E. coli* cells.

The transformants were used to express a fusion protein comprised of SOD at the N-terminus and in-frame C33c HCV antigen at the C-terminus. Expression was accomplished by inoculating 1500 ml of Luria broth containing ampicillin (100 micrograms/ml) with 15 ml of an overnight culture of the transformants. The cells were grown to an O.D. of 0.3, IPTG was added to yield a final concentration of 2 mM, and growth continued until the cells attained a density of 1 O.D., at which time they were harvested by centrifugation at 3,000 x g at 4°C for 20 minutes. The packed cells can be stored at -80°C for several months.

In order to purify the SOD-C33c polypeptide the bacterial cells in which the polypeptide was expressed were subjected to osmotic shock and mechanical disruption, the insoluble fraction containing SOD-C33c was isolated and subjected to differential extraction with an alkaline-NaCl solution, and the fusion polypeptide in the extract purified by chromatography on columns of S-Sepharose and Q-Sepharose.

The crude extract resulting from osmotic shock and mechanical disruption was prepared by the following procedure. One gram of the packed cells were suspended in 10 ml of a solution containing 0.02 M Tris HCl, pH 7.5, 10 mM EDTA, 20% sucrose, and incubated for 10 minutes on ice. The cells were then pelleted by centrifugation at 4,000 x g for 15 min at 4°C. After the supernatant was removed, the cell pellets were resuspended in 10 ml of Buffer A1 (0.01M Tris HCl, pH 7.5, 1 mM EDTA, 14 mM beta-mercaptoethanol [BME]), and incubated on ice for 10 minutes. The cells were again pelleted at 4,000 x g for 15 minutes at 4°C. After removal of the clear supernatant (periplasmic fraction I), the cell pellets were resuspended in Buffer A1, incubated on ice for 10 minutes, and again centrifuged at 4,000 x g for 15 minutes at 4°C. The clear supernatant (periplasmic fraction II) was removed, and the cell pellet resuspended in 5 ml of Buffer A2 (0.02 M Tris HCl, pH 7.5, 14 mM BME, 1 mM EDTA, 1 mM PMSF). In order to disrupt the cells, the suspension (5 ml) and 7.5 ml of Dyno-mill lead-free acid washed glass beads (0.10-0.15 mm diameter)(obtained from Glen-Mills, Inc.) were placed in a Falcon tube, and vortexed at top speed for two minutes, followed by cooling for at least 2 min on ice; the vortexing-cooling procedure was repeated another four times. After vortexing, the slurry was filtered through a scintered glass funnel using low suction; the glass beads were washed two times with Buffer A2, and the filtrate and washes combined.

The insoluble fraction of the crude extract was collected by centrifugation at 20,000 x g for 15 min at 4°C, washed twice with 10 ml Buffer A2, and resuspended in 5 ml of MILLI-Q water.

A fraction containing SOD-C33c was isolated from the insoluble material by adding to the suspension NaOH (2 M) and NaCl (2 M) to yield a final concentration of 20 mM each, vortexing the mixture for 1 minute, centrifuging it 20,000 x g for 20 min at 4°C, and retaining the supernatant.

In order to purify SOD-C33c on S-Sepharose, the supernatant fraction was adjusted to a final concentration of 6M urea, 0.05M Tris HCl, pH 7.5, 14 mM BME, 1 mM EDTA. This fraction was then applied to a column of S-Sepharose Fast Flow (1.5 x 10 cm) which had been equilibrated with Buffer B (0.05M Tris HCl, pH 7.5, 14 mM BME, 1 mM EDTA). After application, the column was washed with two column volumes of Buffer B. The flow through and wash fractions were collected. The flow rate of application and wash, was 1 ml/min; and collected fractions were 1 ml. In order to identify fractions containing SOD-C33c, aliquots of the fractions were analyzed by electrophoresis on 10% polyacrylamide gels containing SDS followed by staining with Coomassie blue. The fractions are also analyzable by Western blots using an antibody directed against SOD. Fractions containing SOD-C33c were pooled.

Further purification of SOD-C33c was on a Q-Sepharose column (1.5 x 5 cm) which was equilibrated with Buffer B. The pooled fractions containing SOD-C33c obtained from chromatography on S-Sepharose was applied to the column. The column was then washed with Buffer B, and eluted with 60 ml of a gradient of 0.0 to 0.4 M NaCl in Buffer B. The flow rate for application, wash, and elution was 1 ml/min; collected fractions were 1 ml. All fractions from the Q-Sepharose column were analyzed as described for the S-Sepharose column. The peak of SOD-C33c eluted from the column at about 0.2 M NaCl.

The SOD-C33c obtained from the Q-Sepharose column was greater than about 90% pure, as judged by analysis on the polyacrylamide SDS gels and immunoblot using a monoclonal antibody directed against human SOD.

Example 2: Synthesis of HCV Antigen C100

HCV antigen C100 contains sequences from the NS3 and NS4 domains. Specifically, it includes amino acids 1569-1931 of Figure 1. This antigen was produced in yeast. A cDNA fragment of a 1270 bp encoding the above amino acids and heaving EcoRI termini was prepared.

The construction of a yeast expression vector in which this fragment was fused directly to the *S. cerevisiae* ADH2/GAP promoter was accomplished by a protocol which included amplification of the C100 sequence using a PCR method, followed by ligation of the amplified sequence into a cloning vector. After cloning, the C100 sequence was excised, and with a sequence which contained the ADH2/GAP promoter, was ligated to a large fragment of a yeast vector to yield a yeast expression vector.

The PCR amplification of C100 was performed using as template the vector pS3-56_{C100m}, which had been linearized by digestion with Sall. pS3-56, which is a pBR322 derivative, contains an expression cassette which is comprised of the ADH2/GAPDH hybrid yeast promoter upstream of the human superoxide dismutase gene, and a downstream alpha factor transcription terminator.

The oligonucleotide primers used for the amplification were designed to facilitate cloning into the expression vector, and to introduce a translation termination codon. Specifically, novel 5'-HindIII and 3'-Sall sites were generated with the PCR oligonucleotides. The oligonucleotide containing the Sall site also encodes the double termination codons, TAA and TGA. The oligonucleotide containing the HindIII site also contains an untranslated leader sequence derived from the pgap63 gene, situated immediately upstream of the AUG codon. The pEco63GAPDH gene is described by Holland and Holland (1980) and by Kniskern et al. (1986). The PCR primer sequences used for the direct expression of C100m were:

```
5' GAG TGC TCA AGC TTC AAA ACA AAA TGG CTC
ACT TTC TAT CCC AGA CAA AGC AGA GT 3'
```

and

```
5' GAG TGC TCG TCG ACT CAT TAG GGG GAA
ACA TGG TTC CCC CGG GAG GCG AA 3'.
```

Amplification by PCR, utilizing the primers, and template, was with a Cetus-Perkin-Elmer PCR kit, and was performed according to the manufacturer's directions. The PCR conditions were 29 cycles of 94°C for a minute, 37°C for 2 minutes, 72°C for 3 minutes; and the final incubation was at 72°C for 10 minutes. The DNA can be stored at 4°C or -20°C overnight.

After amplification, the PCR products were digested with HindIII and Sall. The major product of 1.1 kb was purified by electrophoresis on a gel, and the eluted purified product was ligated with a large Sall-HindIII fragment of pBR322. In order to isolate correct recombinants, competent HB101 cells were transformed with the recombinant vectors, and after cloning, the desired recombinants were identified on the basis of the predicted size of HindIII-Sall fragments excised from the clones. One of the clones which contained the a HindIII-Sall fragment of the correct size was named pBR322/C100⁻d. Confirmation that this clone contained amplified C100 was by direct sequence analysis of the HindIII-Sall fragment.

The expression vector containing C100 was constructed by ligating the HindIII-Sall fragment from pBR322/C100⁻d to a 13.1 kb BamHI-Sall fragment of pBS24.1, and a 1369 bp BamHI-HindIII fragment containing the ADH2/GAP promoter. (The latter fragment is described in EPO 164,556). The pBS24.1 vector is described in commonly owned U.S.S.N. 382,805 filed 19 July 1989. The ADH2/GAP promoter fragment was obtained by digestion of the vector pGAP/AG/HindIII with HindIII and BamHI, followed by purification of the 1369 bp fragment on a gel.

Competent HB101 cells were transformed with the recombinant vectors; and correct recombinants were identified by the generation of a 2464 bp fragment and a 13.1 kb fragment generated by BamHI and Sall digestion of the cloned vectors. One of the cloned correct recombinant vectors was named pC100⁻d#3.

In order to express C100, competent cells of *Saccharomyces cerevisiae* strain AB122 (MATa leu2 ura3-53 prb 1-1122 pep4-3 prcl-407[cir-0]) were transformed with the expression vector pC100⁻d#3. The transformed cells were plated on URA-sorbitol, and individual transformants were then streaked on Leu⁻ plates.

Individual clones were cultured in Leu⁻, ura⁻ medium with 2% glucose at 30°C for 24-36 hours. One liter of Yeast Extract Peptone Medium (YEP) containing 2% glucose was inoculated with 10 ml of the overnight culture, and the resulting culture was grown at 30°C at an agitation rate of 400 rpm and an aeration rate of 1 L of air per 1 L of medium per minute (i.e., 1vvm) for 48 hours. The pH of the medium was not controlled. The culture was grown in a BioFlo II fermentor manufactured by New Brunswick Science Corp. Following fermentation, the cells were isolated and analyzed for C100 expression.

Analysis for expressed C100 polypeptide by the transformed cells was performed on total cell lysates and crude extracts prepared from single yeast colonies obtained from the Leu⁻ plates. The cell lysates and crude extracts were analyzed by electrophoresis on SDS polyacrylamide gels, and by Western blots. The Western blots were probed with rabbit polyclonal antibodies directed against the SOD-C100 polypeptide expressed in yeast. The expected size of the C100 polypeptide is 364 amino acids. By gel analysis the expressed polypeptide has a MW_r of 39.9K.

Both analytical methods demonstrated that the expressed C100 polypeptide was present in total cell lysates, but was absent from crude extracts. These results suggest that the expressed C100 polypeptide may be insoluble.

Example 3: Expression of HCV Antigen S2

HCV antigen S2 contains a sequence from the hydrophobic N-terminus of the S domain. It includes amino acids 199-328 of Figure 1.

The protocol for the construction of the expression vector encoding the S2 polypeptide and for its expression in yeast was analogous to that used for the expression of the C100 polypeptide, described in Example 2.

The template for the PCR reaction was the vector pBR322/Pi14a, which had been linearized by digestion with HindIII. Pi14a is a cDNA clone that encodes amino acids 199-328.

The oligonucleotides used as primers for the amplification by PCR of the S2 encoding sequence were the following. For the 5'-region of the S2 sequence:

5' GAG TGC TCA AGC TTC AAA ACA AAA TGG GGC TCT
ACC ACG TCA CCA ATG ATT GCC CTA AC 3';

and
for the 3'-region of the S2 sequence:

5' GAG TGC TCG TCG ACT CAT TAA GGG GAC CAG TTC
ATC ATC ATA TCC CAT GCC AT 3'.

The primer for the 5'-region introduces a HindIII site and an ATG start codon into the amplified product. The primer for the 3'-region introduces translation stop codons and a Sall site into the amplified product.

The PCR conditions were 29 cycles of 94°C for a minute, 37°C for 2 minutes, 72°C for 3 minutes, and the final incubation was at 72°C for 10 minutes.

The main product of the PCR reaction was a 413 bp fragment, which was gel purified. The purified fragment was ligated to the large fragment obtained from pBR322 digested with HindIII and Sall fragment, yielding the plasmid pBR322/S2d.

Ligation of the 413 bp HindIII-Sall S2 fragment with the 1.36 kb BamHI-HindIII fragment containing the ADH2/GAP promoter, and with the large BamHI-Sall fragment of the yeast vector pBS24.1 yielded recombinant vectors, which were cloned. Correct recombinant vectors were identified by the presence of a 1.77 kb fragment after digestion with BamHI and Sall. An expression vector constructed from the amplified sequence, and containing the sequence encoding S2 fused directly to the ADH2/GAP promoter is identified as pS2d#9.

Example 4: Synthesis of HCV C Antigen

HCV antigen C22 is from the C domain. It comprises amino acids 1-122 of Figure 1.

The protocol for the construction of the expression vector encoding the C polypeptide and for its expression in yeast was analogous to that used for the expression of the C100 polypeptide, described supra, except for the following.

EP 0 693 687 A1

The template for the PCR reaction was pBR322/Ag30a which had been linearized with HindIII. Ag30 is a cDNA clone that encodes amino acids 1-122. The oligonucleotides used as primers for the amplification by PCR of the C encoding sequence were the following.

For the 5'-region of the C sequence :

5' GAG TGC AGC TTC AAA ACA AAA TGA GCA CGA
ATC CTA AAC CTC AAA AAA AAA AC 3' ,

and
for the 3'-region of the C sequence:

5' GAG TGC TCG TCG ACT CAT TAA CCC AAA TTG CGC
GAC CTA CGC CGG GGG TCT GT 3' .

The primer for the 5'-region introduces a HindIII site into the amplified product, and the primer for the 3'-region introduces translation stop codons and a Sall site. The PCR was run for 29 cycles of 94°C for a minute, 37°C for 2 minutes, 72°C for 3 minutes, and the final incubation was at 72°C for 10 minutes.

The major product of PCR amplification is a 381 bp polynucleotide. Ligation of this fragment with the Sall-HindIII large Sall-HindIII fragment of pBR322 yielded the plasmid pBR322/C2.

Ligation of the 381 bp HindIII-Sall C coding fragment excised from pBR322/C2 with the 1.36 kb BamHI-HindIII fragment containing the ADH2/GAP promoter, and with the large BamHI-Sall fragment of the yeast vector pBS24.1 yielded recombinant vectors, which were cloned. Correct recombinant vectors were identified by the presence of a 1.74 kb fragment after digestion with BamHI and Sall. An expression vector constructed from the amplified sequence, and containing the sequence encoding C fused directly to the ADH2/GAP promoter is identified as pC22.

Analysis for expressed C polypeptide by the transformed cells was performed on total cell lysates and crude extracts prepared from single yeast colonies obtained from the Leu⁻ plates. The cell lysates and crude extracts were analyzed by electrophoresis on SDS polyacrylamide gels. The C polypeptide is expected to have 122 amino acids and by gel analysis the expressed polypeptide has a MW_r of approximately 13.6 Kd.

Example 5: Synthesis of NS5 Polypeptide

This polypeptide contains sequence from the N-terminus of the NS5 domain. Specifically it includes amino acids 2054 to 2464 of Figure 1. The protocol for the construction of the expression vector encoding the NS5 polypeptide and for its expression were analogous to that used for the expression of C33c (see Example 1).

Example 6: Radioimmunoassay (RIA) for Antibodies to HCV

The HCV antigens of Examples 1-5 were tested in an RIA format for their ability to detect antibodies to HCV in the serum of individuals clinically diagnosed as having HCV (Non-A, Non-B) and in serum from blood given by paid blood donors.

The RIA was based upon the procedure of Tsu and Herzenberg (1980) in SELECTED METHODS IN CELLULAR IMMUNOLOGY (W.H. Freeman & Co.), pp. 373-391. Generally, microtiter plates (Immulon 2, Removawell strips) are coated with purified HCV antigen. The coated plates are incubated with the serum samples or appropriate controls. During incubation, antibody, if present, is immunologically bound to the solid phase antigen. After removal of the unbound material and washing of the microtiter plates, complexes of human antibody-NANBV antigen are detected by incubation with ¹²⁵I-labeled sheep anti-human immunoglobulin. Unbound labeled antibody is removed by aspiration, and the plates are washed. The radioactivity in individual wells is determined; the amount of bound human anti-HCV antibody is proportional to the radioactivity in the well.

Specifically, one hundred microliter aliquots containing 0.1 to 0.5 micrograms of the HCV antigen in 0.125 M Na borate buffer, pH 8.3, 0.075 M NaCl (BBS) was added to each well of a microtiter plate (Dynatech Immulon 2 Removawell Strips). The plate was incubated at 4°C overnight in a humid chamber, after which, the antigen solution was removed and the wells washed 3 times with BBS containing 0.02% Triton X-100 (BBST). To prevent non-specific binding, the wells were coated with bovine serum albumin (BSA) by addition of 100 microliters of a 5 mg/ml solution of BSA in BBS

EP 0 693 687 A1

followed by incubation at room temperature for 1 hour; after this incubation the BSA solution was removed. The antigens in the coated wells were reacted with serum by adding 100 microliters of serum samples diluted 1:100 in 0.01M Na phosphate buffer, pH 7.2, 0.15 M NaCl (PBS) containing 10 mg/ml BSA, and incubating the serum containing wells for 1 hr at 37°C. After incubation, the serum samples were removed by aspiration, and the wells were washed 5 times with BBST. Antibody bound to the antigen was determined by the binding of ¹²⁵I-labeled F(ab)₂ sheep anti-human IgG to the coated wells. Aliquots of 100 microliters of the labeled probe (specific activity 5-20 microcuries/microgram) were added to each well, and the plates were incubated at 37°C for 1 hour, followed by removal of excess probe by aspiration, and 5 washes with BBST. The amount of radioactivity bound in each well was determined by counting in a counter which detects gamma radiation.

10

15

20

25

30

35

40

45

50

55

EP 0 693 687 A1

Table 1 below presents the results of the tests on the serum from individuals diagnosed as having HCV.

Table 1

5

	<u>INDIVIDUAL</u>	<u>ANTIGEN</u>				
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u>	<u>NS5</u>
10	CVH IVDA	P	P	P(+++)	P	P
	CVH IVDA	P	P	P(+)	P	P
	CVH IVDA	P	P	P(+)	P	P
	CVH NOS	P	P	P	P	P
15	AVH NOS HS	N	N	N	N	N
	AVH NOS HS	P	N	N	N	N
	AVH NOS HS	P	N	N	N	N
	AVH NOS HS	P/N	N	N	N	N
20	AVH PTVH	N	N	N	P/N	N
	AVH NOS HS	N	N	N	N	N
	AVH NOS	N	N	N	N	P
	AVH PTVH	N	N	N	N	N
25	AVH IVDA	N	P	N	P	P
	AVH PTVH	P	P/N	N	N	P
	AVH NOS	N	P	N	N	N
	AVH IVDA	N	P	N	P	P
30	AVH NOS HS	P/N	N	N	N	N
	AVH PTVH	N	N	N	N	N
	CVH IVDA	P	P	P	P	P
	CVH IVDA	P	P	P	P	P
35	AVH NOS HS	N	N	N	N	N
	CVH PTVH	P	P	N	N	N
	AVH PTVH	P	N	P(+)	P(+++)	N
	CVH PTVH	N	P	P	P	P
40	CVH NOS HS	P	P	P	P	N
	CVH NOS	N	P	P/N	P	P
	CVH NOS	N	P	P/N	P	P
	CVH NOS	N	P	P/N	P	P

50

55

EP 0 693 687 A1

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>				
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u>	<u>NS5</u>
5	CVH IVDA	N	N	N	P	N
	AVH IVDA	P	P	P	P	P
	AVH IVDA	P	P	P	P	P
10	CVH IVDA	P	P	P	P	P
	AVH IVDA	P/N	P	N	P	P
	AVH IVDA	N	P	P	P	N
15	CVH PTVH	P	P/N	N	N	N
	CVH NOS	N	N	N	N	N
	CVH NOS	N	N	N	N	N
	CVH IVDA	P	P	P	P	P
20	AVH IVDA	P	P	P	P	P
	CVH PTVH	P	P	P	P	P
	AVH PTVH?	N	P	P	P	P
25	AVH IVDA	N	P	N	P	N
	AVH NOS	N	N	N	N	N
	AVH NOS	N	N	N	N	N
	CVH NOS	N	P	N	N	P
30	CVH NOS	P	P	N	N	N
	CVH NOS HS	P	P	P	P	P
	CVH PTVH	P	P	N	P	P
35	AVH nurse	P	P	N	N	N
	AVH IVDA	P	P	P	P	N
	AVH IVDA	N	P	P(+)	P(+++)	N
40	AVH nurse	P/N	P	N	N	N
	AVH PTVH	P/N	P	P	N	P
	AVH NOS	N	P/N	N	N	P
	AVH NOS	N	P	N	P	N
45	AVH PTVH	P	P/N	N	N	N
	AVH PTVH	N	P	N	P	P
	AVH PTVH	P	P	P	P	P
50	AVH PTVH	N	P	N	N	P
	CVH PTVH	P/N	P	P(+)	P(+++)	N
	AVH PTVH	N	P/N	P(+)	P(+++)	P

55

EP 0 693 687 A1

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
5	AVH PTVH	P	(?)	P	N P
	CVH PTVH	N	P	N	P P
	CVH PTVH	N	P	P	P P
10	CVH PTVH	N	N	N	N N
	AVH NOS	N	N	N	N N
	AVH nurse	P	P	N	N N
15	CVH PTVH	N	P	N	N P
	AVH IVDA	N	P	N	P/N N
	CVH PTVH	P	P	P(+)	P(+++) P
	AVH NOS	P	P	N	N N
20	AVH NOS	P/N	P	N	N P
	AVH PTVH	P/N	P	P	P P
	AVH NOS	N	P	P	P P/N
25	AVH IVDA	N	P	N	N P
	AVH NOS	N	P/N	N	N N
	AVH NOS	P	P	N	N P
	AVH PTVH	N	P	P	P P
30	crypto	P	P	P	P P
	CVH NOS	N	P	P	P P
	CVH NOS	N	N	N	N N
35	AVH PTVH	N	P	P(+)	P(++) N
	AVH PTVH	N	P/N	P(+)	P(++) P
	AVH PTVH	N	P/N	P(+)	P(++) P
40	CVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
45	AVH NOS	N	P	N	N N
	CVH IVDA	P	P	P	P P/N
	AVH IVDA	P	P	P	P N
50	AVH NOS	P	P	N	N N
	AVH NOS	P	P	N	N N
	CVH PTVH	P	P	N	N P/N

55

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
5	AVH PTVH	N	P	N	P P
	AVH NOS	N	N	N	N N
	AVH NOS	N	P	N	N N
10	AVH NOS	P	N	N	N N
	CVH NOS	N	N	N	N N
	AVH NOS	N	P/N	N	N N
15	AVH IVDA	N	P	P	P P
	.crypto	N	P	N	N P/N
	crypto	P	P	P/N	P P
	AVH IVDA	N	N	P	P N
20	AVH IVDA	N	P	P	P N
	AVH NOS	N	N	N	N N
	AVH NOS	N	N	N	N N
25	CVH IVDA	P	P	P	P P
	CVH PTVH	N	N	N	N N
	CVH PTVH	P	P	P(+)	P(+++) P
	CVH PTVH	P	P	P(+)	P(+++) P
30	CVH NOS	P/N	N	N	N N
	CVH NOS	N	N	N	N N
	CVH PTVH	P	P	P	P P
35	CVH PTVH	P	P	P	P P
	CVH PTVH	P	P	P	P P
	AVH IVDA	N	P	P	P P
40	CVH NOS	N	N	N	N N
	CVH NOS	N	N	N	N N
	CVH PTVH	P	P	P	P P
	AVH NOS	P	P	N	N P/N
45	AVH NOS	N	P/N	N	N N
	CVH PTVH	P	P	N	N P
	CVH NOS	N	P/N	N	N N
50	AVH NOS	N	P	N	N N
	AVH NOS	N	P	N	N N
	CVH PTVH	N	P	N	N N

55

EP 0 693 687 A1

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
5	AVH IVDA	N	P	N	P P
	AVH NOS	P	N	N	N N
	CVH NOS	N	N	N	N N
10	CVH NOS	N	N	N	N N
	CVH IVDA	P	P	P	P P
	CVH IVDA	P/N	P	P	P P
15	CVH IVDA	P	P	P	P P
	CVH IVDA	N	P	P	P P
	AVH NOS	N	P	N	N N
	CVH IVDA	N	P	N	N P
20	CVH IVDA	N	P	N	N P
	AVH PTVH	P	P	N	P P
	AVH PTVH	P	P	N	P P
25	CVH NOS	N	P/N	N	N P/N
	CVH NOS	N	P	N	N N
	CVH NOS	N	N	N	N N
	CVH PTVH	P	P	P	P P
30	CVH PTVH	P	P	P	P P
	CVH PTVH	P	P	P	P P
	AVH IVDA	N	P	N	N P
35	AVH IVDA	N	P	P(++)	P(+) P
	CVH PTVH	P	P	P	P P
	AVH PTVH	N	P	P	P P
40	CVH PTVH?	N	P	P	P P
	CVH PTVH?	P/N	P	P	P P
	CVH NOS HS	P	P	N	N N
	CVH IVDA	P	P	P	P N
45	CVH PTVH	N	P	P	P P
	CVH PTVH	P	P	P	P P/N
	CVH NOS	P	P	P	P P
50	CVH IVDA	P	P	P	P P
	CVH PTVH	P	P	P	P N
	CVH PTVH	P	P	P	P P

55

	<u>INDIVIDUAL</u>			<u>ANTIGEN</u>	
	<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u>	<u>NS5</u>
5					
	CVH NOS	N	N	N	P/N
	CVH NOS	N	P/N	N	P/N
10	CVH PTVH	P	P	P	P
	CVH NOS	N	P	N	N
	CVH NOS	N	N	N	N
	CVH NOS	P	P	N	P/N
15	CVH NOS	N	N	N	N
	CVH NOS HS	P	P	P	P
	CVH NOS HS	P	P	P	P
20	CVH PTVH	N	N	N	N
	AVH PTVH	N	P	P	P
	AVH NOS		-	-	
	CVH PTVH	N	P	P(+++)	N
25	crypto	P	P	P	P
	crypto	P	P	P	P
	crypto	N	P	N	N
30	crypto	N	P	P	P
	CVH PTVH	P	P	P	P
	crypto	N	N	N	N
	crypto	N	P	N	P/N
35	crypto	N	P	N	P
	crypto	P	P	P	P
	crypto	N	P	P	N
40	crypto		-	-	
	crypto		-	-	
	CVH NOS		-	-	
45	AVH-IVDA	N	P	P(+)	P

50

55

INDIVIDUALANTIGEN

5		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u>	<u>NS5</u>
	AVH-IVDA	N	P/N	N	P(++)	N

AVH = acute viral hepatitis

10 CVH = chronic viral hepatitis

PTVH = post-transfusion viral hepatitis

IVDA = intravenous drug abuser

15 crypto = cryptogenic hepatitis

-NOS = non-obvious source

P = positive

20 N = negative

25 Per these results, no single antigen reacted with all sera. C22 and C33c were the most reactive and S2 reacted with some sera from some putative acute HCV cases with which no other antigen reacted. Based on these results, the combination of two antigens that would provide the greatest range of detection is C22 and C33c. If one wished to detect a maximum of acute infections, S2 would be included in the combination.

30

35

40

45

50

55

EP 0 693 687 A1

Table 2 below presents the results of the testing on the paid blood donors.

Table 2

5

10

15

20

25

30

35

40

45

50

55

Antigens					
<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
1	N	N	N	N	N
2	N	N	N	N	N
3	P	P	P	P	P
4	N	N	N	N	N
5	N	N	N	N	N
6	N	N	N	N	N
7	N	N	N	N	N
8	N	N	N	N	N

EP 0 693 687 A1

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	9	N	N	N	N	N
	10	N	N	N	N	N
	11	N	N	N	N	N
10	12	N	N	N	N	N
	13	N	N	N	N	N
	14	N	N	N	N	N
15	15	N	N	N	N	N
	16	N	N	N	N	N
	17	N	N	N	N	N
	18	P	P	P	P	P
20	19	P	P	N	P	P
	20	P	P	N	P	P
	21	N	N	N	N	N
25	22	N	P	P	N	P
	23	P	P	P	P	P
	24	N	N	N	N	N
	25	N	N	N	N	N
30	26	N	N	N	N	N
	27	N	N	N	N	N
	28	N	N	N	N	N
35	29	N	N	N	N	N
	30	N	N	N	N	N
	31	P	P	P	N	P
	32	N	N	N	N	N
40	33	N	N	N	N	N
	34	N	N	N	N	P
	35	N	N	P	N	P
45	36	N	N	N	N	N
	37	N	N	N	N	N
	38	N	N	N	N	N
	39	N	N	N	N	N
50	40	N	N	N	N	N
	41	N	N	N	N	P
	42	N	N	N	N	N
55						

EP 0 693 687 A1

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	43	N	N	N	N	N
	44	N	N	N	N	N
	45	N	N	N	N	N
10	46	N	N	N	N	N
	47	P	P	N	N	P
	48	N	N	N	N	N
15	49	N	N	N	N	N
	50	N	N	N	N	N
	51	N	P	P	N	P
	52	N	N	N	N	N
20	53	N	P	P	N	P
	54	P	P	P	P	N
	55	N	N	N	N	N
25	56	N	N	N	N	N
	57	N	N	N	N	N
	58	N	N	N	N	N
	59	N	N	N	N	N
30	60	N	N	N	N	N
	61	N	N	N	N	N
	62	N	N	N	N	N
35	63	N	N	N	N	N
	64	N	N	N	N	N
	65	N	N	N	N	N
40	66	N	N	N	N	N
	67	N	N	N	N	N
	68	N	N	N	N	N
	69	N	N	N	N	N
45	70	P	P	P	P	P
	71	N	N	N	N	N
	72	N	N	N	N	N
50	73	P	P	P	P	N
	74	N	N	N	N	N
	75	N	N	N	N	N
55	76	N	N	N	N	P

EP 0 693 687 A1

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	77	N	N	N	N	N
	78	N	N	N	N	N
	79	N	N	N	N	N
10	80	N	N	N	N	N
	81	N	N	N	N	N
	82	N	N	N	N	N
15	83	P	P	N	N	N
	84	N	N	P	N	N
	85	N	N	N	N	N
	86	P	P	P	P	N
20	87	N	N	N	N	N
	88	N	N	N	N	N
	89	P	P	P	P	P
25	90	P	P	P	P	N
	91	N	N	N	N	P
	92	P	P	P	N	N
	93	N	N	N	N	N
30	94	N	N	N	N	N
	95	N	N	N	N	N
	96	N	N	N	N	N
35	97	N	N	N	N	N
	98	N	P	P	P	P
	99	P	P	P	P	P
40	100	N	N	N	N	N
	101	P	P	P	P	P
	102	N	N	N	N	N
	103	N	N	N	N	N
45	104		N	N	N	N
	105	P	P	P	P	N
	106	N	N	N	N	N
50	107	N	N	N	N	N
	108	N	N	N	N	N
	109	P	P	P	P	P
	110	P	P	P	N	P
55						

EP 0 693 687 A1

		Antigens				
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	111	P	P	P	N	P
	112	N	N	N	N	N
	113	P	P	P	P	P
10	114	N	N	N	N	N
	115	N	N	N	N	N
	116	P	P	P	P	P
15	117	N	N	N	N	N
	118	N	N	N	N	N
	119	N	N	N	N	N
	120	P	P	P	P	P
20	121	N	N	N	N	N
	122	N	P	P	N	P
	123	N	N	N	N	N
25	124	N	N	N	N	N
	125	N	N	N	N	N
	126	P	N	N	N	N
	127	N	N	N	N	N
30	128	N	N	N	N	N
	129	N	N	N	N	N
	130	P	P	P	P	N
35	131	N	N	N	N	P
	132	N	N	N	N	N
	133	N	N	N	N	N
	134	N	N	N	N	N
40	135	N	N	N	N	N
	136	N	N	N	N	N
	137	N	N	N	N	N
45	138	N	N	N	N	N
	139	N	N	N	N	N
	140	P	N	N	N	N
50	141	P	N	P	P	P
	142	N	N	N	N	N
	143	N	N	N	N	N
55	144	N	N	N	N	N

EP 0 693 687 A1

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	145	N	N	N	N	N
	146	N	N	N	N	N
	147	N	N	N	N	N
10	148	N	N	N	N	N
	149	N	N	N	N	N
	150	N	N	N	N	N
15	151	N	N	N	N	N
	152	N	N	N	N	N
	153	N	N	N	N	N
	154	P	P	P	P	P
20	155	N	N	N	N	N
	156	N	N	N	N	N
	157	N	N	N	N	N
25	158	N	N	N	N	N
	159	N	N	N	N	N
	160	N	N	N	N	N
	161	P	P	P	P	P
30	162	N	N	N	N	N
	163	N	N	N	N	N
	164	P	P	P	N	P
35	165	N	N	N	N	N
	166	P	P	P	N	P
	167	N	N	N	N	N
	168	N	N	N	N	N
40	169	N	N	N	N	N
	170	N	N	N	N	N
	171	N	N	N	N	N
45	172	N	N	N	N	N
	173	N	N	N	N	N
	174	N	N	N	N	N
	175	N	N	N	N	N
50	176	N	N	N	N	N
	177	N	N	N	N	P
	178	N	N	N	N	N
55						

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	179	N	N	N	N	N
	180	N	N	N	N	N
	181	N	N	N	N	N
10	182	N	N	N	N	N
	183	P	P	P	P	P
	184	N	N	N	N	N
15	185	N	N	N	N	N
	186	N	N	N	N	N
	187	N	N	N	N	N
	188	N	P	P	N	N
20	189	N	N	N	N	N
	190	N	N	N	N	N
	191	N	N	N	N	N
25	192	N	N	N	N	N
	193	N	N	N	N	N
	194	N	N	N	N	N
	195	N	N	N	N	N
30	196	N	N	N	N	N
	197	N	N	N	N	P
	198	P	P	P	N	N
35	199	N	N	N	N	P
	200	P	P	P	P	N

40

The results on the paid donors generally confirms the results from the sera of infected individuals.

Example 7: ELISA Determinations of HCV Antibodies Using Combination of HCV Antigens

45

Plates coated with a combination of C22 and C33c antigens are prepared as follows. A solution containing coating buffer (50mM Na Borate, pH 9.0), 21 ml/plate, BSA (25 micrograms/ml), C22 and C33c (2.50 micrograms/ml each) is prepared just prior to addition to the Removeawell Immulon I plates (Dynatech Corp.). After mixing for 5 minutes, 0.2ml/well of the solution is added to the plates, they are covered and incubated for 2 hours at 37°C, after which the solution is removed by aspiration. The wells are washed once with 400 microliters wash buffer (100 mM sodium phosphate, pH 7.4, 140 mM sodium chloride, 0.1% (W/V) casein, 1% (W/V) Triton x-100, 0.01% (W/V) Thimerosal). After removal of the wash solution, 200 microliters/well of Postcoat solution (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, 0.1% (w/v) casein, 3% sucrose and 2 mM phenylmethylsulfonylfluoride (PMSF)) is added, the plates are loosely covered to prevent evaporation, and are allowed to stand at room temperature for 30 minutes. The wells are then aspirated to remove the solution, and lyophilized dry overnight, without shelf heating. The prepared plates may be stored at 2-8°C in sealed aluminum pouches with dessicant (3 g Sorb-it™ packs).

50

In order to perform the ELISA determination, 20 microliters of serum sample or control sample is added to a well containing 200 microliters of sample diluent (100 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM EDTA, 0.1% (W/V) Casein, 0.01% (W/V) Thimerosal, 1% (W/V) Triton X-100, 100 micrograms/ml yeast extract). The plates are sealed, and are incubated at 37°C for two hours, after which the solution is removed by aspiration, and the wells are

washed three times with 400 microliters of wash buffer (phosphate buffered saline (PBS) containing 0.05% Tween 20). The washed wells are treated with 200 microliters of mouse anti-human IgG-horse radish peroxidase (HRP) conjugate contained in a solution of Ortho conjugate diluent (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, 50% (v/v) fetal bovine serum, 1% (V/V) heat treated horse serum, 1 mM $K_3Fe(CN)_6$, 0.05% (W/V) Tween 20, 0.02% (W/V) Thimerosal). Treatment is for 1 hour at 37°C, the solution is removed by aspiration, and the wells are washed three times with 400 ml wash buffer, which is also removed by aspiration. To determine the amount of bound enzyme conjugate, 200 microliters of substrate solution (10 mg O-phenylenediamine dihydrochloride per 5 ml of Developer solution) is added. Developer solution contains 50 mM sodium citrate adjusted to pH 5.1 with phosphoric acid, and 0.6 microliters/ml of 30% H_2O_2 . The plates containing the substrate solution are incubated in the dark for 30 minutes at room temperature, the reactions are stopped by the addition of 50 microliters/ml 4N sulfuric acid, and the ODs determined.

In a similar manner, ELISAs using fusion proteins of C22 and C33c, and C22, C33c, and S2 and combinations of C22 and C100, C22 and S2, C22 and an NS5 antigen, C22, C33c, and S2, and C22, C100, and S2 may be carried out.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in the fields of molecular biology, immunology, and related fields are intended to be within the scope of the following claims.

Claims

1. A combination of synthetic hepatitis C viral (HCV) antigens comprising:

- (a) a first HCV antigen from the C domain; and
- (b) at least one additional HCV antigen which is an HCV antigen from the S domain.

2. A combination according to claim 1 which further comprises at least one additional HCV antigen which is:

- (i) an HCV antigen from the NS3 domain;
- (ii) an HCV antigen from the NS4 domain; or
- (iii) an HCV antigen from the NS5 domain.

3. A combination according to claim 2 wherein the additional antigen is from the NS3 domain.

4. The combination of claim 3 wherein the first HCV antigen is C22 and the additional HCV antigen is C33c.

5. A combination according to claim 2 wherein the additional antigen is from the NS4 domain.

6. The combination of claim 5 wherein the first HCV antigen is C22 and the additional HCV antigen is C100.

7. The combination of any one of claims 1 to 6 including HCV antigen S2.

8. A combination of synthetic hepatitis C viral (HCV) antigens comprising:

- (a) a first HCV antigen from the C domain; and
- (b) at least one additional HCV antigen selected from the group consisting of

- (i) an HCV antigen from the NS3 domain;
- (ii) an HCV antigen from the NS4 domain;
- (iii) an HCV antigen from the S domain; and
- (iv) an HCV antigen from the NS5 domain.

9. The combination of any one of claims 1 to 8 wherein the combination is in the form of a fusion polypeptide.

10. The combination of any one of claims 1 to 9 wherein the combination is in the form of said first HCV antigen and said additional antigens bound to a common solid matrix.

11. The combination of claim 10 wherein the solid matrix is the surface of a microtiter plate well, a bead or a dipstick.

12. The combination of any one of claims 1 to 8 wherein the combination is in the form of a mixture of said first HCV antigen and said additional HCV antigen(s).

13. A method for detecting antibodies to hepatitis C virus (HCV) in a mammalian body component suspected of containing said antibodies comprising contacting said body component with the combination of synthetic HCV antigens as defined in any one of claims 1 to 12 under conditions that permit antibody antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

5

14. A kit for carrying out an assay for detecting antibodies to hepatitis C antigen (HCV) in a mammalian body component suspected of containing said antibodies comprising in packaged combination:

10

- (a) the combination of synthetic HCV antigens of any one of claims 1 to 12;
- (b) standard control reagents; and
- (c) instructions for carrying out the assay.

15

20

25

30

35

40

45

50

55

FIG. 1A

-319 CACTCCACCATGAATCACTCCCCCTGTGAGGAACTACTGTCTTCAACGCAGAAAAGCGTCTAG
 GTGAGGTGGTACTTAGTGAGGGGACACTCCTTGATGACAGAAAGTGCGTCTTTCGCAGATC
 -259 CCATGGCGTTAGTATGAGTGTGTCAGCCCTCCAGGACCCCCCTCCCCGGGAGAGCCATA
 GGTAACCGCAATCATACTCACAGACGTCGGAGGTCCTGGGGGGAGGCCCTCTCTCGGTAT
 -199 GTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCTTCTTGGA
 CACCAGACGCCCTTGGCCACTCATGTGGCCCTTAACGGTCTCTGGCCCCAGGAAAGAACCT
 -139 TCAACCCGCTCAATGCCCTGGAGATTGGGCGTGCCCCCGCAAGACTGCTAGCCGAGTAGT
 AGTTGGGCGAGTTACGGACCTCTAAACCCGACGGGGCGTCTTGACGATCGGCTCATCA
 - 79 GTTGGGTCGCGAAAGGCCCTTGTGTAAGTCCCTGATAGGGTGCTTGCAGTGCCCCGGGAG
 CAACCCAGCGCTTTCGGGAACACCATGACGGACTATCCCAACGCTCACGGGGCCCTC
 - 19 GTCTCGTAGACCGTGCACC
 CAGAGCATCTGGCACGTGG
 Arg Thr
 MetSerThrAsnProLysProGlnLysLysAsnLysArgAsnThrAsnArgArgProGln
 1 ATGAGCACGAATCCTAAACCTCAAAAAAACAACAAACGTAACACCAACCGTCGCCACAG
 TACTCGTGCTTAGGATTGGAGTTTTTTTTTTTGTGTCATTGTGGTTGGCAGCGGGTGTC
 AspValLysPheProGlyGlyGlyGlnIleValGlyGlyValTyrLeuLeuProArgArg
 61 GACGTCAAGTTCCTCCGGTGGCGGTGAGATCGTGTGGTGAGTTACTTGTGCCCGCAGG
 CTGCAGTTCAAGGGCCCCACCGCCAGTCTAGCAACCACTCAATGAACAACGGCGCGTCC

121 GlyProArgLeuGlyValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly
 GGGCTAGATTGGGTGTGGCGCGACGAGAAAGACTTCCGAGCGGTGCAACCTCGAGGT
 CCGGATCTAACCCACACGCGCGCTGCTCTTCTGAAGGCTCGCCAGCGTTGGAGCTCCA

 181 ArgArgGlnProIleProLysAlaArgArgProGluGlyArgThrTrpAlaGlnProGly
 AGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCCGAGGCGAGGACCTGGGCTCAGCCCCGG
 TCTGCAGTCGGATAGGGGTTCCGAGCAGCCGGGCTCCCGTCTCGACCCGAGTCGGGGCCC

 241 TyrProTrpProLeuTyrGlyAsnGluGlyCysGlyTrpAlaGlyTrpLeuLeuSerPro
 TACCCCTGGCCCCCTCTATGGCAATGAGGGCTGCGGGTGGGGATGGCTCCTGTCTCCC
 ATGGGAACCGGGGAGATACCGTTACTCCGACGCCACCCGCCCTACCGAGGACAGAGGG

 301 ArgGlySerArgProSerTrpGlyProThrAspProArgArgSerArgAsnLeuGly
 CGTGGCTCTCGGCCCTAGCTGGGGCCCCACAGACCCCCGGCGTAGGTCGCGCAATTGGGT
 GCACCGAGAGCCGGATCGACCCCCGGGTGTCTGGGGCGCGCATCCAGCGCGTTAAACCCA

 361 LysValIleAspThrLeuThrCysGlyPheAlaAspLeuMetGlyTyrIleProLeuVal
 AAGGTCATCGATACCCCTTACGTGGGCTTCGCCGACCTCATGGGTACATACCGCTCGTC
 TTCCAGTAGCTATGGGAATGCACGCCGAAGCGGCTGGAGTACCCCATGTATGGCGAGCAG

 421 GlyAlaProLeuGlyGlyAlaAlaArgAlaLeuAlaHisGlyValArgValLeuGluAsp
 GCGCCCCCTCTTGGAGCGGCTGCCAGGCCCTGGCGCATGGCGTCCGGTCTCTGGAAGAC
 CCGGGGGAGAACCTCCGCGACGGTCCCGGACCGCGTACCGCAGGCCCAAGACCTTCTG

FIG. 1B

FIG. 1C

Thr

481 GlyValAsnTyrAlaThrGlyAsnLeuProGlyCysSerPheSerIlePheLeuLeuAla
 GCGTGAACTATGCAACAGGGAACCTTCCTGGTTGCTCTTCTCTATCTTCTCTGGCC
 CCGCACTTGATACGTTGTCCCTTGGAAAGGACCAACGAGAAAGAGATAGAAAGAACCGG

541 LeuLeuSerCysLeuThrValProAlaSerAlaTyrGlnValArgAsnSerThrGlyLeu
 CTGCTCTCTTGCTTGACTGTGCCCGCTTCGGCCCTACCAAGTGGCAACTCCACGGGCTT
 GACGAGAGAACGAACTGACACGGGCGAAGCCGGATGGTTACGCGTTGAGGTGCCCCGAA

601 TyrHisValThrAsnAspCysProAsnSerSerIleValTyrGluAlaAlaAspAlaIle
 TACCACGTCAACCAATGATTGCCCTAACTCGAGTATTGTGTACGAGCGCGGATGCCATC
 ATGGTGCAGTGGTTACTAACGGGATTGAGCTCATAACACATGCTCCGCGGTACGGTAG

661 LeuHisThrProGlyCysValProCysValArgGluGlyAsnAlaSerArgCysTrpVal
 CTGCACACTCCGGGTGCGTCCCTTGCCTTCGTTCGTGAGGGCAACGCTCGAGGTGTGGTG
 GACGTGTGAGGCCCCACGCAGGGAACGCAAGCACTCCCGTTGCGGAGCTCCACAACCCAC

721 AlaMetThrProThrValAlaThrArgAspGlyLysLeuProAlaThrGlnLeuArgArg
 GCGATGACCCCTACGGTGGCCACCCAGGGATGGCAAACTCCCGCGACGAGCTTCGACGT
 CGCTACTGGGGATGCCACCGGTGGTCCCTACCGTTTGAAGGGCGCTGCGTCAAGCTGCA

781 HisIleAspLeuLeuValGlySerAlaThrLeuCysSerAlaLeuTyrValGlyAspLeu
 CACATCGATCTGCTTGTGCGGAGCGCCACCCCTCTGTTCGGCCCTCTACGTGGGGACCTA
 GTGTAGCTAGACGAACAGCCCTCGCGGTGGGAGACAAGCCGGGAGATGCACCCCTGGAT

841 CysGlySerValPheLeuValGlyGlnLeuPheThrPheSerProArgArgHisTrpThr
 TCGGGTCTGTCTTCTTGTGCGGCCAACTGTTCACCTTCTCTCCAGGCGCCACTGGACG
 ACGCCGAGACAGAAAGAACAGCCGGTTGACAAGTGAAGAGAGGGTCCGCGGTGACCTGC

ThrGlnGlyCysAsnCysSerIleTyrProGlyHisIleThrGlyHisArgMetAlaTrp
 901 ACGCAAGTTGCAATTGCTCTATCTATCCCGGCCATATAACGGGTACCGCATGGCATGG
 TGCCTTCCAACGTTAACGAGATAGATAGGCGCGGTATATTGCCCACTGCGGTACCGTACC

 Val
 AspMetMetMetAsnTrpSerProThrThrAlaLeuValMetAlaGlnLeuLeuArgIle
 961 GATATGATGATGAACCTGGTCCCTACGACGGCGTTGGTAATGGCTCAGCTGCCGATC
 CTATACTACTTGACCAAGGGATGCTGCCGCAACCATTAACGAGTCGACGAGGCCTAG

 ProGlnAlaIleLeuAspMetIleAlaGlyAlaHisTrpGlyValLeuAlaGlyIleAla
 1021 CCACAAGCCATCTTGGACATGATCGCTGGTGCTCACTGGGAGTCTCTGGCGGCATAGCG
 GGTGTTCCGTAGAACCTGTACTAGCGACCAACGAGTGACCCCTCAGGACCGCCCGTATCGC

 TyrPheSerMetValGlyAsnTrpAlaLysValLeuValValLeuLeuPheAlaGly
 1081 TATTTCTCCATGGTGGGAACTGGCGGAAGTCTGGTAGTGCTGCTGCTATTGCCCCGC
 ATAAAGAGGTACCAACCCCTTGACCCGCTTCCAGGACCATCACGACGACGATAAACGGCCG

 ValAspAlaGluThrHisValThrGlyGlySerAlaGlyHisThrValSerGlyPheVal
 1141 GTCGACGCGGAACCCACGTCACCGGGGAAGTGCCGGCCACACTGTGTCTGGATTGTGT
 CAGCTGCCGCTTTGGGTGCAGTGCGCCCCCTTCACGCGCGGTGTGACACAGACCTAAACAA

 SerLeuLeuAlaProGlyAlaLysGlnAsnValGlnLeuIleAsnThrAsnGlySerTrp
 1201 AGCCTCCTCGCACCGCGGCCAAGCAGAACGTCAGCTGATCAACACCAACGCGCAGTTGG
 TCGGAGGAGCGTGGTCCGCGGTTCGTCTTGCAAGTCCGACTAGTTGTGTGTTGCCGTC AAC

FIG. 1D

FIG. 1E

1261 HisLeuAsnSerThrAlaLeuAsnCysAsnAspSerLeuAsnThrGlyTrpLeuAlaGly
 CACCTCAATAGCAGGCCCTGAACCTGCAATGATAGCCTCAACACCGGCTGGTTGGCAGGG
 GTGGAGTTATCGTGCCGGGACTTGACGTTACTATCGGAGTTGTGGCCGACCAACCGTCCC

 1321 LeuPheTyrHisHisLysPheAsnSerSerGlyCysProGluArgLeuAlaSerCysArg
 CTTTCTATACCCACAAGTTCAACTCTTCAGGCTGTCCCTGAGAGGCTAGCCAGCTGCCGA
 GAAAAGATAGTGTGTTCAAGTTGAGAGTCCGACAGGACTCTCCGATCGGTCGACGGCT

 1381 ProLeuThrAspPheAspGlnGlyTrpGlyProIleSerTyrAlaAsnGlySerGlyPro
 CCCCTTACCGATTGTGACCAGGGCTGGGGCCCTATCAGTTATGCCAACGGAACGGCCCC
 GGGGAATGGCTAAAACTGGTCCCGACCCCGGATAGTCAATACGGTTGCCCTTCGCCGGGG

 1441 AspGlnArgProTyrCysTrpHisTyrProProLysProCysGlyIleValProAlaLys
 GACCAGCGCCCTACTGCTGGCACTACCCCCCAAACCTTGGCGTATTGTCCCCCGGAAG
 CTGGTCGCGGGGATGACGACCGGTGATGGGGGTTTGGAAACGCCATAACACGGGCGCTTC

 1501 SerValCysGlyProValTyrCysPheThrProSerProValValGlyThrThrAsp
 AGTGTGTGTGTCGGGTATATTGCTTCACTCCAGCCCCCGTGGTGGGAACGACCCGAC
 TCACACACACAGGCCATATAACGAAGTGAGGTCGGGGCACCAACCCTTGCTGGCTG

 1561 ArgSerGlyAlaProThrTyrSerTrpGlyGluAsnAspThrAspValPheValLeuAsn
 AGTCCGGGCGGCCACCTACAGCTGGGTGAAATGATACGGACGCTCTCGTCCCTAAC
 TCCAGCCCGCGGGTGGATGTCGACCCCACTTTACTATGCTGCTGCAGAACGAGGAATTG

 1621 AsnThrArgProProLeuGlyAsnTrpPheGlyCysThrTrpMetAsnSerThrGlyPhe
 AATACCAGGCCACCGCTGGCAATTGTTTCGGTTGTACCTGGATGAACCTCAACTGGATTC
 TTATGTCCTGGTGGCGACCCGTTAAACCAAGCCCAACATGGACCTACTTGAGTTGACCTAAG

1681 ThrLysValCysGlyAlaProProCysValIleGlyGlyAlaGlyAsnAsnThrLeuHis
 ACCAAAGTGTGGAGCGCCTCCTTGTGTTCATCGGAGGGCGGCAACAACACCTGCAC
 TGGTTTCACACGCCCTCGCGGAGGAACACAGTAGCCTCCCCGCCCTTGTGTGGACGTG

 1741 CysProThrAspCysPheArgLysHisProAspAlaThrTyrSerArgCysGlySerGly
 TGCCCCACTGATTGCTTCCGCAAGCATCCGGACGCCACATACCTCTCGGTGCGCTCCGGT
 ACGGGTGACTAACGAAGCGTTTCGTAGGCTGCGGTGTATGAGAGCCACGCCGAGGCCA

 Ile
 1801 ProTrpLeuThrProArgCysLeuValAspTyrProTyrArgLeuTrpHisTyrProCys
 CCTGGATCACACCCAGGTGCTGTCGACTACCCGTATAGGCTTGGCATATCCTTGT
 GGGACCTAGTGTGGTCCACGGACCGCTGATGGGCATATCCGAAACCGTAATAGGAACA

 1861 ThrIleAsnTyrThrIlePheLysIleArgMetTyrValGlyGlyValGluHisArgLeu
 ACCATCAACTACACCATATTTAAATCAGGATGTACGTGGAGGGTCTGAAACACAGGCTG
 TGGTAGTTGATGTGGTATAAAATTTTAGTCCTACATGCACCCCTCCCTGTGTCTCCGAC

 1921 GluAlaAlaCysAsnTrpThrArgGlyGluArgCysAspLeuGluAspArgAspArgSer
 GAAGCTGCCCTGCAACTGGACCGGGCGAACCCTTGCATCTGGAAGACACAGGACAGGTCC
 CTTCGACGGACGTTGACCTGCGCCCCCTTGCAACGCTAGACCTTCTGTCTCCCTGTCCAGG

 1981 GluLeuSerProLeuLeuLeuThrThrThrGlnTrpGlnValLeuProCysSerPheThr
 GAGCTCAGCCCGTTACTGCTGACCACTACACAGTGGCAGGTCTCCCGTGTCTCTCACA
 CTCGAGTCGGGCAATGACGACTGGTGTGTGTACCGTCCAGGAGGGCACAAAGGAAGTGT

FIG. 1F

FIG. 1G

2041 ThrLeuProAlaLeuSerThrGlyLeuIleHisLeuHisGlnAsnIleValAspValGln
 ACCCTACCAGCCTTGTCACCGCCTCATCCACCTCCACAGAACATTTGTGGACGTGCAG
 TGGGATGGTCGGAACAGGTGGCCGGAGTAGGTGGAGTGGTCTTGTAAACACCTGCACGTC

 2101 TyrLeuTyrGlyValGlySerSerIleAlaSerTrpAlaIleLysTrpGluTyrValVal
 TACTTGTACGGGTGGGTCAAGCATCGCGTCTCTGGCCATTAAAGTGGAGTACGTCGTT
 ATGAACATGCCCCACCCAGTTCGTAGCGCAGGACCCGGTAATTCAACCTCATGCAGCAA

 2161 LeuLeuPheLeuLeuAlaAspAlaAargValCysSerCysLeuTrpMetMetLeuLeu
 CTCCTGTTCCCTTCTGCTTGACAGACGCGCGTCTGCTCCTGCTTGTGGATGATGCTACTC
 GAGGACAAAGGAGACGAACGTCTGCGCGCAGACGAGGACGAACACCTACTACGATGAG

 2221 IleSerGlnAlaGluAlaAlaLeuGluAsnLeuValIleLeuAsnAlaAlaSerLeuAla
 ATATCCCAAGCGGAGCGGCTTGGAGAACCTCGTAATACTTAATGCAGCATCCCTGGCC
 TATAGGGTTCGCCCTCCGCCGAAACCTCTTGGAGCATTATGAATTACGTCTGTAGGGACCGG

 2281 GlyThrHisGlyLeuValSerPheLeuValPhePheCysPheAlaTrpTyrLeuLysGly
 GGGACGCACGGTCTTGATCCTTCCCTCGTGTCTTCTGCTTGCATGGTATTTGAAGGGT
 CCCTGCGTGCCAGAACATAGGAAGGAGCACAAAGAACGAAACGTACCATAACTTCCCA

 2341 LysTrpValProGlyAlaValTyrThrPheTyrGlyMetTrpProLeuLeuLeuLeu
 AAGTGGGTGCCCGAGCGGTCTACACCTTCTACGGGATGTGGCCTCTCCTCCTCCTTG
 TTCACCCACGGGCCCTCGCCAGATGTGGAAGATGCCCTACACCGGAGAGGACGAGGAC

 2401 LeuAlaLeuProGlnArgAlaTyrAlaLeuAspThrGluValAlaAlaSerCysGlyGly
 TTGGCGTTGCCCCAGCGGCGTACGCGCTGGACACGAGGTGGCCGCTCGTGTGGCGGT
 AACCGCAACGGGTGCGCCCGCATGCGCGACCTGTGCTCCACCGGCGCAGCACACCGCCA

2461 ValValLeuValGlyLeuMetAlaLeuThrLeuSerProTyrTyrLysArgTyrIleSer
 GTTGTCTCTCGTGGGTTGATGGCGCTGACTCTGTCAACATATTACAAGCGCTATATCAGC
 CAACAAGAGCAGCCCCAACTACCGGACTGAGACAGTGGTATAATGTTCCGGATATAGTCG

 2521 TrpCysLeuTrpTrpLeuGlnTyrPheLeuThrArgValGluAlaGlnLeuHisValTrp
 TGGTGCTTGTGGCTTCAGTATTTCTGACCAAGAGTGGAAAGCGAACTGCACGTGTGG
 ACCACGAACACACCGAAGTCATAAAAGACTGGTCTCACCTTCGCGTTGACGTGCACACC

 2581 IleProProLeuAsnValArgGlyGlyArgAspAlaValIleLeuLeuMetCysAlaVal
 ATTCCCCCCTCAACGTCGAGGGGGCGGACGCCGTCACTTACTCATGTGTGCTGTA
 TAAGGGGGGAGTTGCAGGCTCCCCCGCTGCGGCAGTAGAATGAGTACACACGACAT

 2641 HisProThrLeuValPheAspIleThrLysLeuLeuAlaValPheGlyProLeuTrp
 CACCCGACTCTGGTATTGACATCACCAAATTGCTGCTGGCCGTCTTCGGACCCCTTTGG
 GTGGGCTGAGACCATAAACTGTAGTGGTTAAACGACGCCGAGAACCTGGGGGAAACC

 2701 IleLeuGlnAlaSerLeuLeuLysValProTyrPheValArgValGlnGlyLeuLeuArg
 ATTCTTCAAGCCAGTTTGCTTAAAGTACCCTACTTTGTGCGCGTCCAAGGCCCTTCTCCGG
 TAAGAAAGTTCGGTCAAAACGAATTTCATGGGATGAAACACGCCGACAGTTCCGGAAAGGCC

 2761 PheCysAlaLeuAlaArgLysMetIleGlyGlyHisTyrValGlnMetValIleIleLys
 TTCTGCGCGTTAGCGGGAAGATGATCGGAGGCCATTACGTGCAAAATGGTCATCATTAAG
 AAGACGCGCAATCGCGCCTTCTACTAGCCTCCGGTAATGCACGTTTACCAGTAGTAATTC

FIG. 1H

FIG. 11

2821 LeuGlyAlaLeuThrGlyThrTyrValTyrAsnHisLeuThrProLeuArgAspTrpAla
 TTAGGGCGCTTACTGGACCTATGTTTATAAACCATCTCACTCCTCTTCGGGACTGGGCG
 AATCCCCGCGAATGACCGTGATACAAATATTGGTAGAGTGAGGAGAAGCCCTGACCCGC

 2881 HisAsnGlyLeuArgAspLeuAlaValAlaValGluProValValPheSerGlnMetGlu
 CACAACGGCTTGGAGATCTGGCCGTGGCTGTAGAGCCAGTCGTCTTCTCCAAATGGAG
 GTGTTGCCGAACGCTCTAGACCGGCACCGACATCTCGGTGAGCAGAAAGGGTTACCTC

 2941 ThrLysLeuIleThrTrpGlyAlaAspThrAlaAlaCysGlyAspIleIleAsnGlyLeu
 ACCAAGCTCATCACGTGGGGGCAGATACCGCCGTCGTGGGTGACATCATCAACGGCTTG
 TGGTTCGAGTAGTGCAACCCCCGCTCTATGGCGGCACGCCCACTGTAGTAGTTGCCGAAC

 3001 ProValSerAlaArgArgGlyArgGluIleLeuLeuGlyProAlaAspGlyMetValSer
 CCTGTTTCCGCCCGCAGGGCGGGAGATACTGCTCGGGCCAGCCGATGGAATGGTCTCC
 GGACAAAGCGGGCTCCCCGGCCCTCTATGACGAGCCCCGGTCGGCTACCTTACCAGAGG

 3061 LysGlyTrpArgLeuLeuAlaProIleThrAlaTyrAlaGlnGlnThrArgGlyLeuLeu
 AAGGGTGGAGGTTGCTGGCGCCCCATCACGGCGTACGCCCAGCAGACAAAGGGCCCTCCTA
 TTCCCCACCTCCAAACGACCGCGGGTAGTGCCGCATGCGGGTCTGTGTCCCCGGAGGAT

 3121 GlyCysIleIleThrSerLeuThrGlyArgAspLysAsnGlnValGluGlyGluValGln
 GGGTGCAATAATCACACGCCCTAACTGGCCGGGACAAACCAAGTGGAGGTGAGGTCCAG
 CCCACGTATTAGTGGTCGGATTGACCGGCCCTGTTTGTGTTACCTCCCACTCCAGGTC

 3181 IleValSerThrAlaAlaGlnThrPheLeuAlaThrCysIleAsnGlyValCysTrpThr
 ATGTGTCAACTGCTGCCCAAAACCTTCTCTGGCAACGTGCATCAATGGGTGTGCTGGACT
 TAACACAGTTGACGACGGGTTTGGAAAGGACCGTTGCACGTAGTTACCCCAACACGACCTGA

3241 ValTyrHisGlyAlaGlyThrArgThrIleAlaSerProLysGlyProValIleGlnMet
 GTCTACCACGGGCGGAAACGAGGACCATCGCGTCAACCAAGGTCTCTCATCCAGATG
 CAGATGGTGCCCCCGCCTTGCTCCTGTAGCGCAGTGGGTCCAGGACAGTAGGTCTAC

3301 TyrThrAsnValAspGlnAspLeuValGlyTrpProAlaProGlnGlySerArgSerLeu
 TATACCAATGTAGACCAAGACCTTGTTGGCTGGCCCGCTCCGCAAGTAGCCGCTCATTG
 ATATGGTTACATCTGGTTCTGGAACACCCGACCGGGCGAGCGTTCCTATCGGCGAGTAAC

3361 ThrProCysThrCysGlySerSerAspLeuTyrLeuValThrArgHisAlaAspValIle
 ACACCCCTGCACTTGCGGCTCCTCGGACCTTTACCTGGTCACGAGGCACGCCGATGTCATT
 TGTGGGACGTGAACGCCGAGGAGCCTGGAATGGACCAAGTCTCCGTCCGGCTACAGTAA

3421 ProValArgArgGlyAspSerArgGlySerLeuLeuSerProArgProIleSerTyr
 CCCGTGCGCGCGGGTGATAGCAGGGCAGCCTGTGTGTCGCCCGCCCATTTCCCTAC
 GGGCACGCGCGCCCACTATCGTCCCCGTCCGACGACAGCGGGCGCGGTAAAGGATG

3481 LeuLysGlySerSerGlyGlyProLeuLeuCysProAlaGlyHisAlaValGlyIlePhe
 TTGAAAGGCTCCTCGGGGGTCCGCTGTGTGCCCCCGGGGCACGCCGTGGCATATTT
 AACTTCCGAGGAGCCCCCAGGGCACAACACGGGGCGCCCCGTGCGGCACCCGTATAAA

3541 ArgAlaAlaValCysThrArgGlyValAlaLysAlaValAspPheIleProValGluAsn
 AGGGCCGCGGTGTCACCCGTGGAGTGGCTAAGCGGTGACTTTATCCCTGTGGAGAAC
 TCCCCGGGCCACACGTGGGCACCTCACCGATTCCGCCACCTGAAATAGGGACACCTCTTG

FIG. 1J

FIG. 1K

3601 LeuGluThrThrMetArgSerProValPheThrAspAsnSerSerProProValValPro
 CTAGAGACAAACCATGAGGTCCCGGTGTTCAAGGATAACTCTCTCCACCAAGTAGTGCCC
 GATCTCTGTTGGTACTCCAGGGCCACAAAGTGCCCTATTGAGGAGAGGTGGTCATCACGGG

 3661 GlnSerPheGlnValAlaHisLeuHisAlaProThrGlySerGlyLysSerThrLysVal
 CAGAGCTTCCAGGTGGCTCACCTCCATGCTCCACAGGACGGCAAAAGCACCAAGGTC
 GTCTCGAAGTCCACCGAGTGGAGGTACGAGGGTGTCCTCGCCGTTTTCGTGGTTCCAG

 3721 ProAlaAlaTyrAlaAlaGlnGlyTyrLysValLeuValLeuAsnProSerValAlaAla
 CCGGCTGCATATGCAGCTCAGGGCTATAAGGTGCTAGTACTCAACCCCTCTGTGCTGCA
 GGCCGACGTATACGTCGAGTCCCGATATTCCACCATCATGAGTTGGGGAGACAAACGACGT

 3781 ThrLeuGlyPheGlyAlaTyrMetSerLysAlaHisGlyIleAspProAsnIleArgThr
 AACTGGGCTTTGGTGCTTACATGTCCAAAGGCTCATGGGATCGATCCTAACATCAGGACC
 TGTGACCCGAAACCAACGAAATGTACAGGTTCCGAGTACCCCTAGCTAGGATTGTAGTCCTGG

 3841 GlyValArgThrIleThrThrGlySerProIleThrTyrSerThrTyrGlyLysPheLeu
 GGGTGAGAAACAATTACCACTGGCAGCCCCCATCACGTACTCCACCTACGGCAAGTTCCTT
 CCCCACCTCTGTTAATGGTGACCGTCGGGGTAGTGTCATGAGGTGGATGCCGTTCAAGGAA

 3901 AlaAspGlyGlyCysSerGlyGlyAlaTyrAspIleIleIleCysAspGluCysHisSer
 GCCGACGGCGGTGCTCGGGGGCGCTTATGACATAATAATTGTGACGAGTGCCACTCC
 CGGCTGCCGCCCCACGAGCCCCCGCGAATACTGTATTATTAAACACTGCTCACGGTGAGG

3961 ThrAspAlaThrSerIleLeuGlyIleGlyThrValLeuAspGlnAlaGluThrAlaGly
 (Val)
 ACGATGCCACATCCATCTTGGGACATCGGCACTGCTCTTGACCAAGCAGAGACTGCCGGG
 TGCCTACGGTGTAGGTAGAACCCGTAGCCGTGACAGGAAGTGGTTCGTCTCTGACGCCCC
 4021 AlaArgLeuValValLeuAlaThrAlaThrProProGlySerValThrValProHisPro
 GCGAGACTGGTTGTGCTCGCCACCGCCACCCCTCCGGCTCCGTCACTGTGCCCATCCC
 CGCTCTGACCAACACAGCGGTGGCGGTGGGAGGCCCGAGGCAGTGACACGGGGTAGGG
 4081 AsnIleGluGluValAlaLeuSerThrThrGlyGluIleProPheTyrGlyLysAlaIle
 AACATCGAGGAGGTGCTCTGTCCACCAACCGGAGAGATCCCTTTTACGGCAAGCTATC
 TTGTAGCTCCTCCAACGAGACAGGTGGTGGCTCTCTAGGAAATGCGTTCGATAG
 4141 ProLeuGluValIleLysGlyGlyArgHisLeuIlePheCysHisSerLysLysCys
 CCCCTCGAAGTAATCAAGGGGGGAGACATCTCATCTTCTGTGCATTCAAAGAGAAAGTGC
 GGGAGCTTCATTAGTTCCTCCCTCTGTAGAGTAGAAGACAGTAAGTTCTTCTTCACG
 4201 AspGluLeuAlaAlaLysLeuValAlaLeuGlyIleAsnAlaValAlaTyrTyrArgGly
 GACGAACTCGCCGCAAGCTGGTCGCAATGGGCATCAATGCCGTGGCCCTACTACCGCGGT
 CTGCTTGAGCGGGCTTCGACACGCGTAACCCGTAGTTACGGCACCGGATGATGGCGCCA
 4261 LeuAspValSerValIleProThrSerGlyAspValValValAlaThrAspAlaLeu
 CTTGACGTGTCGGTCATCCCGACCAAGCGGCGATGTTGTCGTGCGCAACCGATGCCCTC
 GAACTGCACAGGCAGTAGGGCTGGTCCGCGCTACAAACAGCAGCACCCGTTGGCTACGGGAG
 4321 MetThrGlyTyrThrGlyAspPheAspSerValIleAspCysAsnThrCysValThrGln
 Tyr
 ATGACCGGCTATACCGCGACTTCGACTCGGTGATAGACTGCAATACGTGTGTACCCAG
 TACTGGCCGATATGGCCGCTGAAGCTGAGCCACTATCTGACGTTATGCACACAGTGGGTC

FIG. 1L

FIG. 1M

(Ser)

ThrValAspPheSerLeuAspProThrPheThrIleGluThrIleThrLeuProGlnAsp
 4381 ACAGTCGATTTCAGCCTTGACCCCTACCTTACCATTCAGACAATCACGCTCCCCCAGGAT
 TGTCAAGCTAAAGTCGGAACTGGGATGGAAGTGTAACCTCTGTAGTCGCGAGGGGTCCTA

 AlaValSerArgThrGlnArgArgGlyArgThrGlyArgGlyLysProGlyIleTyrArg
 4441 GCTGTCTCCCCGCACTCAACGTCGGGGCAGGACTGGCAGGGGGAAGCCAGGCATCTACAGA
 CGACAGAGGGCGTGAGTTGCAGCCCCCGTCCCTGACCGTCCCCCTTCGGTCCGTAGATGTCT

 PheValAlaProGlyGluArgProSerGlyMetPheAspSerSerValLeuCysGluCys
 4501 TTTGTGGCACCGGGGAGCGCCCCCTCCGGCATGTTTCGACTCGTCCGTCTCTGTGAGTGC
 AAACACCGTGGCCCCCTCGCGGGGAGGCCGTACAAGCTGAGCAGGCGAGGACACTCACG

 TyrAspAlaGlyCysAlaTrpTyrGluLeuThrProAlaGluThrThrValArgLeuArg
 4561 TATGACGCAGGCTGTGCTTGGTATGAGCTCACGCCCGCCGAGACTACAGTTAGGCTACGA
 ATACTGCGTCCGACACGAAACCATACTCGAGTGGCGGGCTCTGTATGTCAATCCGATGCT

 AlaTyrMetAsnThrProGlyLeuProValCysGlnAspHisLeuGluPheTrpGluGly
 4621 GCGTACATGAACACCCCGGGGCTTCCCGTGTGCCAGGACCATCTTGAATTTTGGGAGGGC
 CGCATGTACTTGTGGGGCCCCCGAAGGCACACGGTCCCTGGTAGAACTTAAACCCCTCCCG

 ValPheThrGlyLeuThrHisIleAspAlaHisPheLeuSerGlnThrLysGlnSerGly
 4681 GTCTTTACAGGCCTCACTCATATAGATGCCCACTTCTATCCAGACAAAGCAGAGTGGG
 CAGAAATGTCCGGAGTGAGTATATCTACGGGTGAAAGATAGGGTCTGTTTCGTCTCACCC

 GluAsnLeuProTyrLeuValAlaTyrGlnAlaThrValCysAlaArgAlaGlnAlaPro
 4741 GAGAACCTTCCTTACCTGGTAGCGTACCAAGCCACCGTGTGGCTAGGGCTCAAGCCCCT
 CTCTTGGAAAGGAATGGACCATCGCATGGTTCGGTGGCACACGCGATCCCAGATTCTGGGA

4801 ProProSerTrpAspGlnMetTrpLysCysLeuIleArgLeuLysProThrLeuHisGly
 CCCCATCGTGGGACCATGTGGAAGTGTTCGATTCGCCCTCAAGCCACCCCTCCATGGG
 GGGGTAGCACCCCTGGTCTACACCTTCACAACTAAGCGGAGTTCGGGTGGGAGGTACCC

 4861 ProThrProLeuLeuTyrArgLeuGlyAlaValGlnAsnGluIleThrLeuThrHisPro
 CCAACACCCCTGCTATACAGACTGGCGCTGTTTCAGAAATGAAATCACCCCTGACGCCACCCA
 GGTGTGGGACGATATGTCTGACCCCGACAAAGTCTTACTTAGTGGGACTGCGTGGGT

 4921 ValThrLysTyrIleMetThrCysMetSerAlaAspLeuGluValValThrSerThrTrp
 GTCACCAAATACATCATGACATGCATGTCGGCCGACCTGGAGTCTGTCAACAGACACCTGG
 CAGTGGTTTATGTAGTACTGTACGTACAGCCGGCTGGACCTCCAGCAGTGTCTCGTGGACC

 4981 ValLeuValGlyGlyValLeuAlaAlaLeuAlaAlaTyrCysLeuSerThrGlyCysVal
 GTGCTCGTTGGCGCGTCCCTGGCTGCTTTGGCCCGGTATTGCCCTGTCAACAGGCTGCGTG
 CACGAGCAACCGCCGACGAGACCGACGAAACCGGGCGCATAACGGACAGTTGTCCGACGCAC

 5041 ValIleValGlyArgValValLeuSerGlyLysProAlaIleIleProAspArgGluVal
 GTCATAGTGGCAGGGTCGTCTGTCCGGGAGCCGGCAATCATATACCTGACAGGGAAGTC
 CAGTATCACCCGTCCTCCAGCAGAACAGGCCCTTCGGCCCTTAGTATGGACTGTCCCTTCAG

 5101 LeuTyrArgGluPheAspGluMetGluGluCysSerGlnHisLeuProTyrIleGluGln
 CTCTACCGAGAGTTTCGATGAGATGGAAGAGTGTCTCTCAGCACTTACCGTACATCGAGCAA
 GAGATGGCTCTCAAGCTACTCTACCTTCTCAGCAGAGTCGTGAATGGCATGTAGCTCGTT

FIG. 1N

FIG. 10

5161 GlyMetMetLeuAlaGluGlnPheLysGlnLysAlaLeuGlyLeuLeuGlnThrAlaSer
 GGGATGATGCTCGCCGAGCAGTTCAAGCAGAAAGCCCTCGGCCCTCCTGCAGACCGCGTCC
 CCTACTACGAGCGGCTCGTCAAGTTCGTCTTCCGGGAGCCGGAGGACGTCTGGCGCAGG

 5221 ArgGlnAlaGluValIleAlaProAlaValGlnThrAsnTrpGlnLysLeuGluThrPhe
 CGTCAGGCAGAGGTTATCGCCCCCTGCTGTCCAGACCAACTGGCAAAACTCGAGACCTTC
 GCAGTCCGCTCTCCAATAGCGGGGACGACAGGTCTGGTTGACCGTTTTTTGAGCTCTGGAAAG

 5281 TrpAlaLysHisMetTrpAsnPheIleSerGlyIleGlnTyrLeuAlaGlyLeuSerThr
 TGGCGGAAGCATATGTGGAACCTTCATCAGTGGGATACAATACTTGGCGGGCTTGTCAACG
 ACCCGCTTCGTATACACCTTGAGTAGTCAACCTATGTATGTAACCGCCCGAACAGTTGC

 5341 LeuProGlyAsnProAlaIleAlaSerLeuMetAlaPheThrAlaAlaValThrSerPro
 CTGCCCTGGTAACCCCGCCATTGCTTCATTGATGGCTTTTACAGCTGTGTCAACGCCCCA
 GACGGACCATTTGGGGCGGTAAACGAAGTAACTACCGAAAAATGTTCGACGACAGTGGTCGGGT

 5401 LeuThrThrSerGlnThrLeuLeuPheAsnIleLeuGlyGlyTrpValAlaAlaGlnLeu
 CTAACCACTAGCCAAACCCCTCCTCTCAACATATTGGGGGGTGGGTGGCTGCCCCAGCTC
 GATTGGTGATCGGTTTGGGAGGAGAAAGTTGTATAACCCCCCACCCACCGGGTCGAG

 5461 AlaAlaProGlyAlaAlaThrAlaPheValGlyAlaGlyLeuAlaGlyAlaAlaIleGly
 GCCGCCCCCGGTGCCGCTACTGCCCTTGTGGCGCTGGCTTAGCTGGCGCCGCCATCGGC
 CGCGGGGGCCACGGCGATGACGGAAACACCCCGGACCGAATCGACCGCGGGGTAGCCG

 5521 SerValGlyLeuGlyLysValLeuIleAspIleLeuAlaGlyTyrGlyAlaGlyValAla
 AGTGTGGACTGGGGAAGTCCCTCATAGACATCCTTGCAGGGTATGGCGCGCGGTGGCG
 TCACAACTGACCCCTTCCAGGAGTATCTGTAGGAACGTCCCATACCGCGCCCGCACCGC

5581 GlyAlaLeuValAlaPheLysIleMetSerGlyGluValProSerThrGluAspLeuVal
 (Gly)
 GGAGCTCTTGTTGGCATTCAAGATCATGAGCGGTGAGGTCCCTCCACGGAGGACCTGGTC
 CCTCGAGAACACCGTAAGTTCTAGTACTCGCCACTCCAGGGAGGTGCCTCCTGGACCCAG

5641 AsnLeuLeuProAlaIleLeuSerProGlyAlaLeuValValGlyValValCysAlaAla
 AATCTACTGCCCGCCATCCTCTCGCCCGAGCCCTCGTAGTCGGCGTGTCTGTGCAGCA
 TTAGATGACGGCGGTAGGAGAGCGGGCCTCGGGAGCATCAGCCGCCACAGACACGTCGT

5701 IleLeuArgArgHisValGlyProGlyGluGlyAlaValGlnTrpMetAsnArgLeuIle
 ATACTGCGCGCGCACGTTGGCCCGGCGAGGGGCAGTGCAGTGGATGAACCGGCTGATA
 TATGACGGCGCGGTGC AACCGGGCCCGCTCCCCGTACCGTCACCTACTTGGCCGACTAT

5761 AlaPheAlaSerArgGlyAsnHisValSerProThrHisTyrValProGluSerAspAla
 GCCTTCGCCTCCCGGGGAACCATGTTTCCCCCACCGCACTACGTGCCGGAGACCGATGCA
 CGGAAGCGGAGGGCCCCCTTGGTACAAAGGGGTGCGTGATGCACGGCCCTCTCGCTACGT

5821 AlaAlaArgValThrAlaIleLeuSerSerLeuThrValThrGlnLeuLeuArgArgLeu
 GGTGCCCGCGTCACTGCCATACTCAGCAGCCTCACTGTAAACCCAGCTCCTGAGGCGACTG
 CGACGGCGCAGTGACGGTATGAGTCGTCGGAGTGACATTGGGTCGAGGACTCCCGCTGAC

5881 HisGlnTrpIleSerSerGluCysThrThrProCysSerGlySerTrpLeuArgAspIle
 CACCAGTGGATAAGCTCGAGTGTACCACTCCATGCTCCGGTTCTCTGGCTAAGGGACATC
 GTGGTCACCTATTTCGAGCCCTCACATGGTGAGGTACGAGGCCAAGGACCGATTCCCTGTAG

FIG. 1P

FIG. 1Q

5941 TrpAspTrpIleCysGluValLeuSerAspPheLysThrTrpLeuLysAlaLysLeuMet
 TGGACTGGATATGCGAGGTGTGAGCGACTTTAAGACCTGGCTAAGCTAAGCTCATG
 ACCCTGACCTATACGCTCCACAACCTCGCTGAAATTCTGGACCGATTTCGATTCCGAGTAC

 6001 ProGlnLeuProGlyIleProPheValSerCysGlnArgGlyTyrLysGlyValTrpArg
 CCACAGCTGCCCTGGGATCCCCCTTTGTGTCCTGCCAGCGCGGTATAAAGGGGTCTGGCGA
 GGTGTCGACGGACCCCTAGGGGAAACACAGGACGGTCGCGCCCATATTCCCCCAGACCGCT
 (Val)
 6061 GlyAspGlyIleMetHisThrArgCysHisCysGlyAlaGluIleThrGlyHisValLys
 GTGGACGGCATCATGCACACTCGCTGCCACTGTGGAGCTGAGATCAGTGGACATGTCAAA
 CACCTGCCGTAGTACGTGTGAGCGACGGTGACACCTCGACTCTAGTGACCTGTACAGTTT

 6121 AsnGlyThrMetArgIleValGlyProArgThrCysArgAsnMetTrpSerGlyThrPhe
 AACGGGACGATGAGGATCGTCGGTCCTAGGACCTGCAGGAACATGTGGAGTGGACCTTC
 TTGCCCTGCTACTCCTAGCAGCCAGGATCCTGGACGTCCTTGTAACACCTCACCCCTGGAAG

 6181 ProIleAsnAlaTyrThrThrGlyProCysThrProLeuProAlaProAsnTyrThrPhe
 CCCATTAAATGCCCTACACCGGGCCCCCTGTACCCCCCTTCTGCGCCGAACTACACGTTT
 GGGTAATTACGGATGTGGTGCCCGGGACATGGGGGAAGGACGCGGCTTGATGTGCAAG

 6241 AlaLeuTrpArgValSerAlaGluGluTyrValGluIleArgGlnValGlyAspPheHis
 GCGCTATGGAGGGTGTCTGCAGAGGAATATGTGGAGATAAGGCAGGTGGGGACTTCCAC
 CGGATACCTCCACAGACGTCTCCTTATACACCTCTATTCCGTCCACCCCCCTGAAGGTG

 6301 TyrValThrGlyMetThrThrAspAsnLeuLysCysProCysGlnValProSerProGlu
 TACGTGACGGGTATGACTACTGACAAATCTCAAATGCCCGTGCAGGTCCCATCGCCCGAA
 ATGCACTGCCCATACTGATGACTGTAGAGTTTACGGGCACGGTCCAGGGTAGCGGGCTT

6361 PhePheThrGluLeuAspGlyValArgLeuHisArgPheAlaProProCysLysProLeu
 TTTTTCACAGAAATTGGACGGGTGCGCTACATAGGTTTGCGCCCCCTGCAAGCCCTTG
 AAAAAGTGCTCTTAACCTGCCCCACGCGGATGATCCAAACGCGGGGACGTTCGGGAAC

 6421 LeuArgGluGluValSerPheArgValGlyLeuHisGluTyrProValGlySerGlnLeu
 CTGCGGAGGAGGTATCATTCAGAGTAGGACTCCACGAATACCCGTTAGGTGCGCAATTA
 GACGCCCTCCTCCATAGTAAGTCTCATCTGAGGTGCTTATGCGCCATCCACGCGTTAAT

 6481 ProCysGluProGluProAspValAlaValLeuThrSerMetLeuThrAspProSerHis
 CCTTGCGAGCCCGAACCGGACGTGGCCGTGTTGACGTCCATGCTCACTGATCCCTCCCAT
 GGAAACGCTCGGGCTTGCCCTGCACCCGGCACAACTGCAGGTACGAGTACTAGGGAGGGTA

 6541 IleThrAlaGluAlaAlaGlyArgArgLeuAlaArgGlySerProProSerValAlaSer
 ATAACAGCAGAGCGCGCGCGGAAGTTGGCGAGGGGATCACCCCCCTCTGTGGCCAGC
 TATTGTCTCTCCGCGCGCGCTTCCAAACCGCTCCCTAGTGGGGGAGACACCGGTCTG

 6601 SerSerAlaSerGlnLeuSerAlaProSerLeuLysAlaThrCysThrAlaAsnHisAsp
 TCCTCGGCTAGCCAGCTATCCGCTCCATCTCTCAAGGCAACTTGCAACCGTAACCATGAC
 AGGAGCCGATCGGTCGATAGGCGAGGTAGAGAGTTCCGTTGAACGTGGCGATTGGTACTG

 6661 SerProAspAlaGluLeuIleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGlyAsn
 TCCCCGTGATGCTGAGCTCATAGAGGCCAACCTCCTATGGAGGCAGGAGATGGGCGGCAAC
 AGGGGACTACGACTCGAGTATCTCCGGTTGGAGGATACCTCCGTCTCTACCCGCCGTTG

FIG. 1R

FIG. 1S

IleThrArgValGluSerGluAsnLysValValIleLeuAspSerPheAspProLeuVal
 6721 ATCACAGGTTGAGTCAGAAACAAAGTGGTGATTCTGGACTCCTTCGATCCGCTTG
 TAGTGGTCCCAACTCAGTCTTTGTTTACCACCTAAGACCTGAGGAAGCTAGGCGAACAC

 AlaGluGluAspGluArgGluIleSerValProAlaGluIleLeuArgLysSerArgArg
 6781 GCGGAGGAGGACGAGCGGGAGATCTCCGTACCCGCAGAAATCCTGCGGAAGTCTCGGAGA
 CGCTCCTCCTGCTCGCCCTCTAGAGGCATGGCGCTCTTTAGGACGCCCTTCAGAGCCCTCT

 PheAlaGlnAlaLeuProValTrpAlaArgProAspTyrAsnProProLeuValGluThr
 6841 TTCGCCAGGCCCTGCCCGTTTGGCGCGCGGACTATAACCCCCGCTAGTGGAGACG
 AAGCGGTCCGGACGGGCAACCCGCGCGGCTGATATTGGGGGGCGGATCACCTCTGC

 TrpLysLysProAspTyrGluProProValValHisGlyCysProLeuProProLys
 6901 TGGAAAAAGCCCGACTACGAACCACTGTGTCCATGGCTGTCCGCTTCCACCTCCAAAG
 ACCTTTTTCGGGCTGATGCTTGGTGACACCAAGGTACCGACAGCGGAAGGTGGAGTTTC

 SerProValProProProArgLysLysArgThrValValLeuThrGluSerThrLeu
 6961 TCCCCCTCCTGTGCCTCCGCCCTCGGAAGAACGCGACGGTGTCTCTCACTGAATCAACCTA
 AGGGAGGACACGGAGGCGGAGCCCTTCTTCGCCCTGCCACCAAGAGTACTAGTTGGGAT

 SerThrAlaLeuAlaGluLeuAlaThrArgSerPheGlySerSerSerThrSerGlyIle
 7021 TCTACTGCCCTTGGCCGAGCTCGCCACCAAGAGCTTTGGCAGCTCCTCACTTCCGGCAT
 AGATGACGGAAACCGGCTCGAGCGGTGCTCTTCGAAACCGTCGAGGAGTTGAAGGCCGTAA

 ThrGlyAspAsnThrThrThrSerSerGluProAlaProSerGlyCysProProAspSer
 7081 ACGGGCGACAATACGACAACATCCTCTGAGCCCCGCCCTTCTGGCTGCCCCCGACTCC
 TGCCCCGCTGTATGCTGTGTAGGAGACTCGGGCGGGGAAGACCGACGGGGGGCTGAGG

(PheAla)

7141 AspAlaGluSerTyrSerSerMetProProLeuGluGlyGluProGlyAspProAspLeu
 GACGCTGAGTCCTATTCTCTCCATGCCCTCCCTGGAGGGAGCCTGGGATCCGGATCTT
 CTGCGACTCAGGATAAGGAGGTACGGGGGGACCTCCCTCGGACCCCTAGGCCTAGAA

7201 SerAspGlySerTrpSerThrValSerSerGluAlaAsnAlaGluAspValValCysCys
 AGCGACGGGTCAATGGTCAACGGTCAGTAGTGAGGCCAACCGGAGGATGTCGTGTGCTGC
 TCGCTGCCAGTACCAAGTTGCCAGTCATCACTCCGGTTGCGCCTCCTACAGCACACGACG

7261 SerMetSerTyrSerTrpThrGlyAlaLeuValThrProCysAlaAlaGluGluGlnLys
 TCAATGTCCTTACTCTTGACAGGCGCACTCGTCAACCCCGTGCCTCGGAGAAACAGAAA
 AGTTACAGAAATGAGAACCTGTCCCGGTGAGCAGTGGGGCACGCGGCCCTTCTGTCTTT

7321 LeuProIleAsnAlaLeuSerAsnSerLeuLeuArgHisHisAsnLeuValTyrSerThr
 CTGCCCCATCAATGCACCTAAGCAACTCGTTGCTACGTCAACCAATTTGGTGTATTCACCC
 GACGGGTAGTTACGTGATTTCGTTGAGCAACGATGCAGTGTGTTAAACCAATAAGGTGG

7381 ThrSerArgSerAlaCysGlnArgGlnLysLysValThrPheAspArgLeuGlnValLeu
 ACCTCACGCACTGCTTGCCAAAGGCAGAGAAAGTCACATTTGACAGACTGCAGTTCTG
 TGGAGTGGGTCAACGAAACGGTTTCCGTCTTCTTTCAGTGTAACCTGTCTGACGTTCAAGAC

7441 AspSerHisTyrGlnAspValLeuLysGluValLysAlaAlaAlaSerLysValLysAla
 GACAGCCATTACCAGGACGTACTCAAGGAGGTTAAAGCAGCGCGCTCAAAGTGAAGGCT
 CTGTCGGTAATGGTCCTGCATGAGTTCCTCCAAATTTTCGTGCGCCGAGTTTTCACACTCCGA

FIG. 1T

FIG. 1U

(Phe)

7501 AsnLeuLeuSerValGluGluAlaCysSerLeuThrProProHisSerAlaLysSerLys
AACTTGCTATCCGTAGAGGAAGCTTGACGCTGACGCCCCACACTCAGCCAAATCCAAG
TTGAACGATAGGCATCTCCTTCGAACGTCGACTCGGGGTGTAGTCGGTTTAGGTTT

7561 PheGlyTyrGlyAlaLysAspValArgCysHisAlaArgLysAlaValThrHisIleAsn
TTTGTTATGGGCAAAAGACGTCCTTGCCATGCCAGAAAGCCGTAACCCACATCAAC
AAACCAATACCCCGTTTTCTGCAGGCAACGGTACGGTCTTTCCGGCATTTGGGTAGTTG

7621 SerValTrpLysAspLeuLeuGluAspAsnValThrProIleAspThrThrIleMetAla
TCCGTGTGGAAGACCTTCTGGAAGACAATGTAACACCAATAGACACTACCATCATGGCT
AGGCACACCTTTCTGTGAAGACCTTCTGTACATTGTGGTTATCTGTGATGGTAGTACCGA

7681 LysAsnGluValPheCysValGlnProGluLysGlyGlyArgLysProAlaArgLeuIle
AAGAACGAGGTTTCTGCGTTCAGCCTGAGAAAGGGGTCTGTAAAGCCAGCTCGTCTCATC
TTCTTGCTCCAAAGACGCAAGTCGGACTCTTCCCCCAGCATTCGGTCGAGCAGAGTAG

7741 ValPheProAspLeuGlyValArgValCysGluLysMetAlaLeuTyrAspValValThr
GTGTTCCCCGATCTGGCGGTGCGCGTGTGCGAAAGATGGCTTTGTACGACGTGGTTACA
CACAAAGGGCTAGACCCGACGCGCACACGCTTTTCTACCGAAACATGCTGCACCAATGT

7801 LysLeuProLeuAlaValMetGlySerSerTyrGlyPheGlnTyrSerProGlyGlnArg
AAGCTCCCTTGCGCGTGATGGGAAGCTCCTACGGATTCCAATACTACCCAGGACAGCGG
TTCGAGGGGAACCGGCACCTACCCCTTCGAGGATGCCTAAGGTTATGAGTGGTCCCTGTCGCC

7861 ValGluPheLeuValGlnAlaTrpLysSerLysLysThrProMetGlyPheSerTyrAsp
GTTGAATTCTCTCGTGCAAGCGTGGAAAGTCCAAAGAAACCCCAATGGGGTTCTCGTATGAT
CAACTTAAGGAGCACGTTCCGACCTTCAGGTTCTTTTGGGGTTACCCCAAGAGCATACTA

7921 ThrArgCysPheAspSerThrValThrGluSerAspIleArgThrGluGluAlaIleTyr
 ACCCGCTGCTTTGACTCCACAGTCACTGAGAGCGACATCCGTACGGAGGAGCAATCTAC
 TGGCGACGAAACTGAGGTGTCACTGACTCTCGCTGTAGGCATGCCTCCTCCGTTAGATG

 7981 GlnCysCysAspLeuAspProGlnAlaArgValAlaIleLysSerLeuThrGluArgLeu
 CAATGTTGTGACCTCGACCCCAAGCCCGCGTGGCCATCAAGTCCCTCACCGAGAGGCTT
 GTTACAACACTGGAGCTGGGGGTTCTGGGGCGCACCCGGTAGTTCAGGGAGTGCTCTCCGAA

 8041 TyrValGlyGlyProLeuThrAsnSerArgGlyGluAsnCysGlyTyrArgArgCysArg
 TATGTTGGGGCCCTCTTACCAATTCAAGGGGGAGAACTGCGGCTATCGCAGGTGCCCG
 ATACAACCCCGGAGAAATGGTTAAGTTCCCCCTCTTGTACGCCGATAGCGTCCACGGCG

 8101 AlaSerGlyValLeuThrThrSerCysGlyAsnThrLeuThrCysTyrIleLysAlaArg
 GCGAGCGCGTACTGACAACTAGCTGTGGTAAACACCTCCTCCTGCTACATCAAGGCCCG
 CGCTCGCCGCATGACTGTTGATCGACACCACTTGTGGGAGTGAAACGATGTAGTTCCGGGCC

 8161 AlaAlaCysArgAlaAlaGlyLeuGlnAspCysThrMetLeuValCysGlyAspAspLeu
 GCAGCCTGTCGAGCCGCGAGGCTCCAGGACTGCACCATGCTCGTGTGTGGCGACGACTTA
 CGTCGGACAGCTCGGCGTCCCGAGGTCCCTGACGTGTACGTGACACACACCGCTGCTGAAT

 8221 ValValIleCysGluSerAlaGlyValGlnGluAspAlaAlaSerLeuArgAlaPheThr
 GTCGTTATCTGTGAAGCGCGGGGTCCAGGAGGACCGCGCGAGCCTGAGAGCCTTCACG
 CAGCAATAGACACTTTCGCGCCCCCAGGTCTCTCTGCGCGCTCGGACTCTCGGAAGTGC

FIG. 1V

FIG. 1W

8281 GluAlaMetThrArgTyrSerAlaProProGlyAspProProGlnProGluTyrAspLeu
 GAGGCTATGACCAGGTACTCCGCCCCCTGGGACCCCCACAAACCAGAAATACGACTTG
 CTCCGATACTGGTCCATGAGCGGGGGGACCCCTGGGGGTGTTGGTCTTATGCTGAAC

 8341 GluLeuIleThrSerCysSerSerAsnValSerValAlaHisAspGlyAlaGlyLysArg
 GAGCTCATAACATCATGCTCCTCCAACGTGTCACTCGCCCCACGACGGCGCTGGAAGAGG
 CTCGAGTATTGTAGTACGAGGAGGTGCACAGTCAGCGGGTGTGCCCGACCTTTCTCC

 8401 ValTyrTyrLeuThrArgAspProThrThrProLeuAlaArgAlaAlaTrpGluThrAla
 GTCTACTACCTCACCCGTGACCCCTACAACCCCTCGCGAGAGCTGCGTGGGAGACAGCA
 CAGATGATGGAGTGGCACTGGGATGTTGGGGGAGCGCTCTCGACGACCCCTCTGTCTCGT

 8461 ArgHisThrProValAsnSerTrpLeuGlyAsnIleIleMetPheAlaProThrLeuTrp
 AGACACACTCCAGTCAATTCTCTGGCTAGGCAACATAATCATGTGTTGCCCCCACACTGTGG
 TCTGTGTGAGGTCAAGTAAAGGACCGATCCGTTGTATTAGTACAAACGGGGGTGTGACACC

 8521 AlaArgMetIleLeuMetThrHisPheSerValLeuIleAlaArgAspGlnLeuGlu
 GCGAGGATGATACTGATGACCCATTCTTTAGCGTCCCTTATAGCCAGGACCCAGCTTGAA
 CGCTCCTACTATGACTACTGGGTAAAGAAATCGCAGGAATATCGGTCCCTGGTCGAACTT

 8581 GlnAlaLeuAspCysGluIleTyrGlyAlaCysTyrSerIleGluProLeuAspLeuPro
 CAGGCCCTCGATTGCGAGATCTACGGGCGCTGCTACTCCATAGAACCACTTGATCTACCT
 GTCCGGGAGCTAACGCTCTAGATGCCCCCGGACCATGAGGTATCTTGGTGAAC TAGATGGA

 8641 ProIleIleGlnArgLeuHisGlyLeuSerAlaPheSerLeuHisSerTyrSerProGly
 CCAATCATTCAAAGACTCCATGGCCCTCAGCGCATTTTCACTCCACAGTTACTCTCCAGGT
 GGT TAGTAAGTTTCTGAGGTACCGGAGTCGCGGTAAAGTGAGGTGTCAATGAGAGGTCCA

8701 GluIleAsnArgValAlaAlaCysLeuArgLysLeuGlyValProProLeuArgAlaTrp
 GAAATTAATAGGTGGCCGATGCCCTCAGAAACCTGGGTACCGCCCTTGCAGCTTGG
 CTTAATTATCCACCGCGGTACGGAGTCTTTGAACCCCATGGCGGAACGCTCGAACC

 Gly
 8761 ArgHisArgAlaArgSerValArgAlaArgLeuAlaArgGlyGlyArgAlaAlaIle
 AGACACCGGGCCCGAGCGTCCGGCTAGGCTTCTGGCCAGAGGAGCGGCTGCCATA
 TCTGTGGCCCGGCTCGCAGCGCGATCCGAAGACCGGTCTCTCCGTCCCGACGGTAT

 8821 CysGlyLysTyrLeuPheAsnTrpAlaValArgThrLysLeuLysLeuThrProIleAla
 TGTGGCAAGTACCTCTTCAACTGGGCAGTAAGAAACAAGCTCAAACTCACTCCAAATAGCG
 ACACCGTTTCATGGAGAAAGTTGACCCGTCATTCTTGTTCGAGTTTGAGTGAGGTTATCGC

 8881 AlaAlaGlyGlnLeuAspLeuSerGlyTrpPheThrAlaGlyTyrSerGlyGlyAspIle
 GCCGCTGGCCAGCTGGACTTGTCCGGCTGGTTACGGCTGGCTACAGCGGGGAGACATT
 CGCGACCGGTCGACCTGAACAGGCCGACCAAGTGCCGACCGATGTGCCCCCTCTGTAA

 8941 TyrHisSerValSerHisAlaArgProArgTrpIleTrpPheCysLeuLeuLeuAla
 TATCACAGCGTGTCTCATGCCCGGCCCGCTGGATCTGGTTTTCCTACTCCTGCTTGCT
 ATAGTGTCGACACAGAGTACGGGGCGGGCGACCTAGACCAAAACGGATGAGGACGAACGA

FIG. 1X

FIG. 1Y

9001 AlaGlyValGlyIleTyrLeuLeuProAsnArgOP
 GCAGGGGTAGGCATCTACCTCCTCCCCAACCCGATGAAGTTGGGGTAAACACTCCGGCCT
 CGTCCCCATCCGTAGATGGAGGGGTTGGCTACTTCCAACCCCATTTGTGAGGCCGGA

() = Heterogeneity due possibly to 5' or 3'-
 terminal cloning artefact

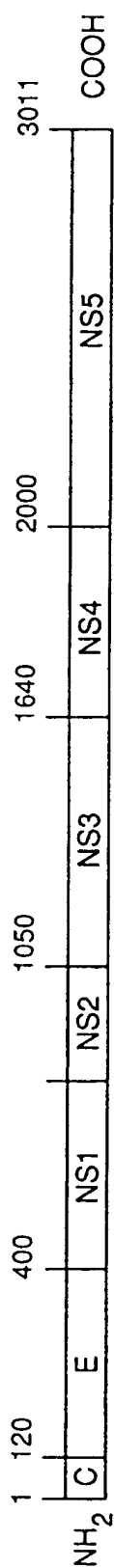


FIG. 2



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 11 4016

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	WO-A-89 04669 (CHIRON CORPORATION) * page 123, line 29 - page 125, line 22 * * page 132, line 3 - page 134, line 35 * * page 171, line 4 - line 20 * ---	1-14	G01N33/576 C07K14/18
A,D	EP-A-0 318 216 (CHIRON CORPORATION) * the whole document * -----	1-14	
			TECHNICAL FIELDS SEARCHED (Int.Cl.5)
			G01N C07K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 16 November 1995	Examiner Van Bohemen, C
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 03.92 (P04 C01)